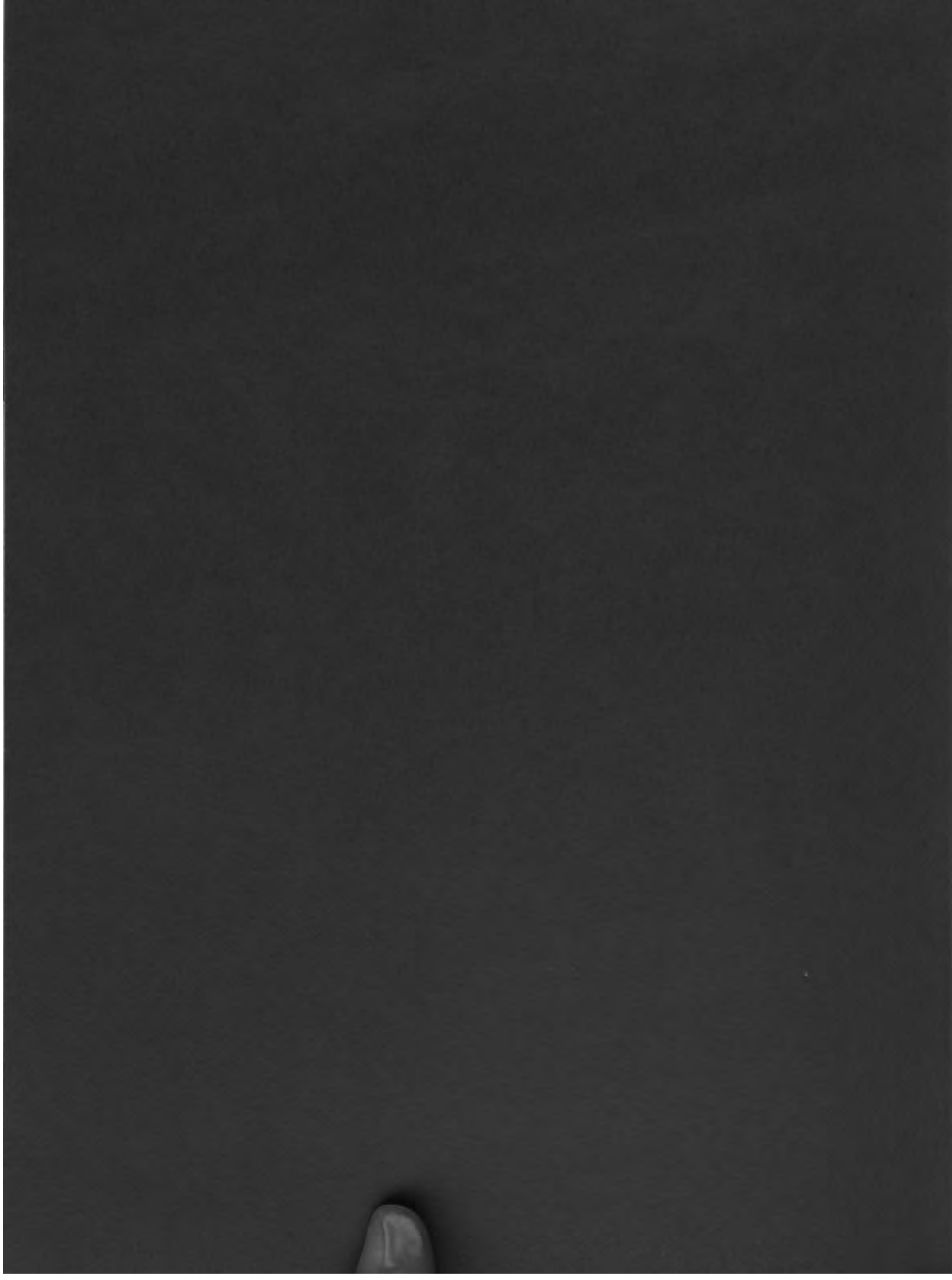
LABORATORY DIAGNOSIS BY SEROLOGIC METHODS

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES / PUBLIC HEALTH SERVICE / CENTERS FOR DISEASE CONTROL / ATLANTA, GEORGIA 30333



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OCTOBER 1975
U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
CENTERS FOR DISEASE CONTROL
LABORATORY IMPROVEMENT PROGRAM OFFICE
LABORATORY TRAINING AND CONSULTATION DIVISION
Atlanta, Georgia 30333

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Diagnostic Immunology Training Branch

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CONTENTS

A. Safety Pipetting

Safety Pipetting Devices

Many pipetting outfits have been designed to protect the laboratorian from mouth contact with toxic or infectious materials. Some examples of these devices, with instructions on their operation, are included here for examination and practice. The technician should be familiar with these devices and adept in the use of at least one of them, as infectious or toxic materials must never be mouth-pipetted.

I. Pipetting Devices

A. **Propipettes**, Safety Pipette Filler - Commercially available through Fisher and others
   1. Operation steps
      a) Press on agate valve "A" and squeeze bulb to expel air and create a vacuum.
      b) Press on agate valve "S" which draws liquid to the desired level.
      c) Press on agate valve "E" to expel liquid. To deliver the last drop, cover air inlet in front of "E", press "E" and squeeze the small bulb on the side.

B. **Caulfield Pipettor** - Commercially available through Fisher and others
   1. Operation steps
      a) Rub glycerine, with fingertip, on face of rubber bushing at bottom of Pipettor. Grip pipette well up at the top and insert firmly into Pipettor.
      b) Squeeze the bulb between thumb and second and third fingers, holding index finger above and away from the control button.
      c) Press down with tip of index finger on the valve control at top of bulb. Then, holding the finger firmly down on top of the bulb will automatically seal the air vent. Place pipette into solution.
      d) To draw up solution keep tip of index finger firmly over control valve, at the same time allowing bulb to open gradually.
      e) To release the fluid accurately, rest tip of index finger on top and a little to the side of the valve control button. Roll the finger gently over onto control button to release liquid. Pipette may be emptied quickly by pressing finger firmly on valve control and squeezing the bulb.

C. **Rubber Bulbs** - Commercially available through Scientific Products
   1. This safety device is difficult to control for accuracy, but is used to draw the fluid into the pipette. The bulb is then removed and the fingertip is used in the usual manner to deliver accurate volumes.

D. **Rubber Tip Device** - Improvised at CDC
   1. This device is not practical with pipettes larger than the 1.0 ml size.
   2. The rubber tip device is used for mixing and, with practice, accurate volumes can be delivered by controlling the angle in which the tube is bent.

II. Safety Devices for Demonstration Only

A. **Fisher Safety Pipette Filler**

B. **Clinac Pipettor** - Lapine Scientific Company

C. **Svedmeyer** - Schick X-ray Company

D. **Pumpette Automatic Pipettor** - Lapine Scientific Company

E. **Cornwall**, Continuous Pipetting Outfit - Becton, Dickinson and Company

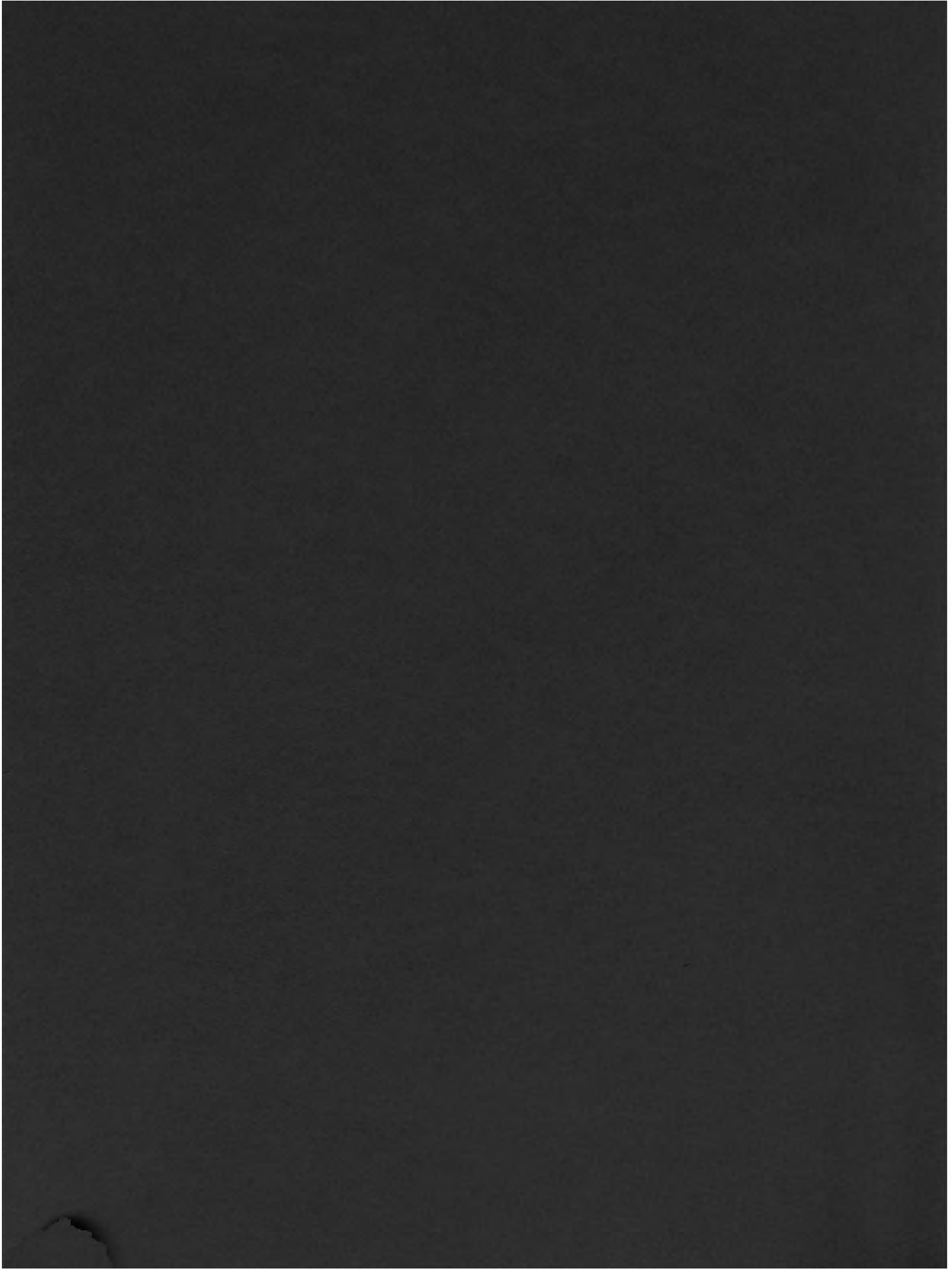
F. **Fisher Pipet Adapter**

G. **Plunger Activated Safety Pipette** - Demuth Glass Division

H. **Eppendorf Automatic Pipets** - Arthur H. Thomas Company

I. **Nalgene Pipetting Aids** - Nalge Sybron Corporation, Nalgene Labware Division

B. Standardization of
Erythrocyte Suspensions
by Spectrophotometer



Standardization of Erythrocyte Suspensions by Spectrophotometer

The spectrophotometric method of standardizing erythrocyte suspensions is applicable and is recommended for all serologic tests employing red blood cells.

I. Calibration of Spectrophotometer

A. Mechanical Operation of Instrument

1) Place the instrument permanently away from direct light and in a vibration-free location.
2) Keep the optical parts of the instrument as free from dirt and dust as possible. (Keep covered)
3) Refer to the manufacturer's manual for other important points concerning line voltage fluctuation, the exciter lamp, and the use of a didymium standard for wavelength calibration.
4) Study the instrument and the attached diagram of the instrument.
5) Locate all the operating controls.

B. Zero Transmittance Adjustment

1) Place line power ON-OFF switch in ON position. Allow the instrument to warm up at least one-half hour.
2) Rotate **COARSE** control to approximately a full scale indication–position the index spot near 100% T or near the value to be used; the fine control may be in any position, preferably midrange.
3) Raise and rotate the cuvet adapter so that the key of the adapter is at right angles to the cuvet well (5) keyway. Cover cuvet well with light shield.
4) The instrument should read exactly 0% transmittance (black scale).
5) If it is not exactly 0% transmittance, adjust with the ZERO control lever (8, located under the machine). This is a coarse adjustment device and may not adjust to exactly 0% transmittance.
6) If necessary, adjust by sliding Scale Panel (3) (fine adjustment) left or right until the zeros on each scale coincide exactly.

C. Wavelength Calibration with Didymium Standard

NOTE: The procedures outlined below always must be followed after *installation* or *transportation* of the instrument and upon *disturbance* or *replacement* of the exciter lamps.

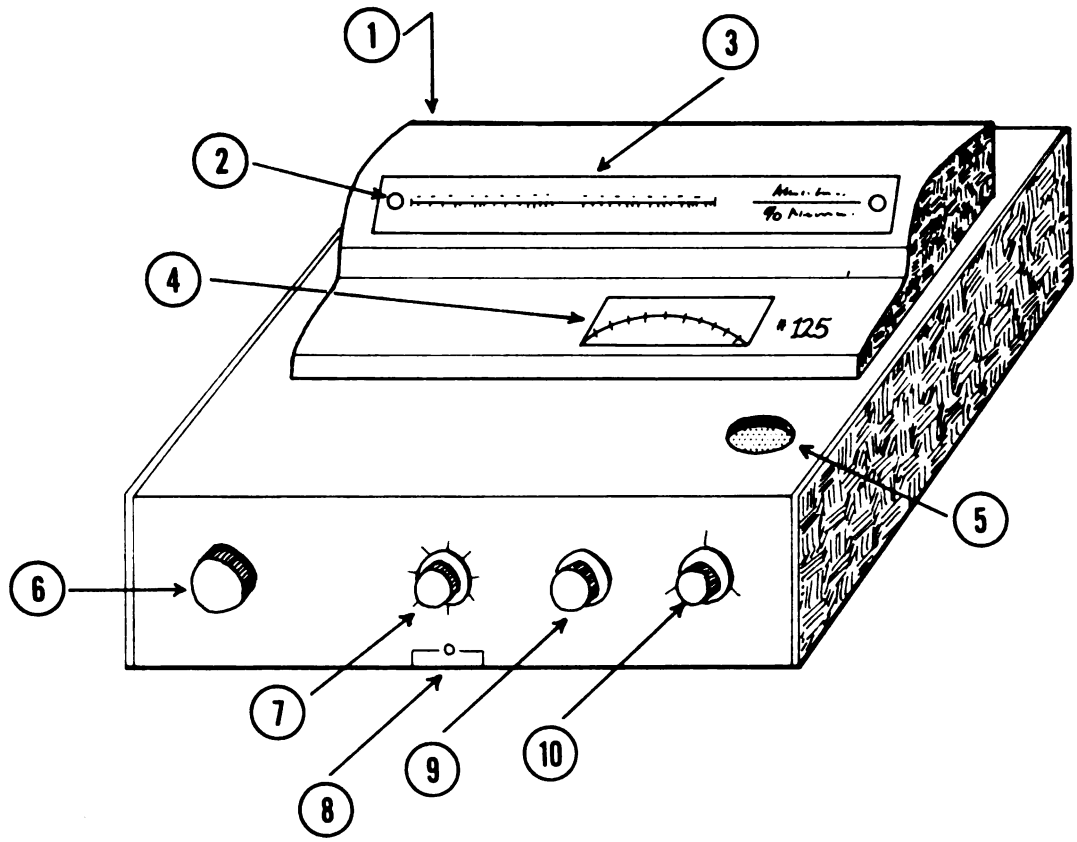
1) Place line power ON-OFF switch in ON position and allow 30 minutes for warm-up of the instrument.
2) Set the wavelength scale to read exactly 610 nanometers (nm; formerly measured in millimicrons).
3) Adjust filter selector (10) to proper position for the desired wavelength range according to the list below:

Position	Wavelength Range mu (nm)	Wavelength Dial Area Color
UV	325-380	Blue
VIS	380-650	White
NIR	650-825	Red

Adjust to visible (VIS) range.

4) Check zero transmittance adjustment as above.
5) Remove cuvet adapter and cover cuvet well opening.
6) Adjust the **COARSE** and **FINE** control knobs so the meter needle points to 100% on the transmittance (black) scale.

SPECTROPHOTOMETER: PARTS IDENTIFICATION



1. Line Power On-Off Switch (Rear Instrument Panel)
2. Friction Spring
3. Scale Panel
4. Wavelength Dial
5. Cuvet Well – covered by cylindrical light shield
6. Wavelength Control
7. Coarse Control
8. Zero Lever Control
9. Fine Control
10. Filter Selector

7) Clean surface of the didymium calibrating standard thoroughly before using it by wiping with clean cleansing tissue, gently blowing off any lint.
8) Uncover the cuvet well and place the clean didymium calibrating standard in the well so the key enters the cuvet well keyway. Make sure the calibrating standard is fully seated in the well.
9) Read and record the % transmittance (black scale) of the didymium calibrating standard.
10) Remove the calibrating standard; insert an opaque piece of white paper into the cuvet well. Determine and record color of the light entering the well.
11) The wavelength calibration of the instrument is correct within 1 nm and is satisfactory if:
    a) The indicated transmittance is within ± 1.5 scale divisions of the value of transmittance (T) marked on the standard **AND**
    b) The light entering the cuvet well is orange.
12) If the wave length calibration is incorrect, see the operating manual which comes with the instrument for correction procedures.

D. Calibrate a series of tubes to obtain a set of matched cuvettes*:

1) Warm up the spectrophotometer a minimum of 10 minutes and set the wavelength at 540 mu.
2) Pipette 4 ml of undiluted cyanmethemoglobin standard into a clean, unscratched cuvette. (Use a plastic or neoprene-coated test tube rack to prevent scratching the tubes.) Wipe the outside of the cuvette clean.
3) Place the cuvette in the sample well of the instrument. Some cuvettes have an etched line at the lip which should be aligned with the mark on the sample well.
4) Adjust with the Coarse and Fine adjustments until the standard reads 50% T. If a "zero adjust" control is present, use it to set the pointer at 0% T when the cuvette holder is empty and covered.
5) Pour the standard solution into the next cuvette to be calibrated.
6) Rotate this tube in the cuvette holder until it reads as near to 50% T as possible. If it will not read within ± 0.5% T, discard the tube.
7) If it falls within ± 0.5% T, etch a mark on the tube at the point where the reading is nearest 50% T. The etching should line up with the mark on the sample well.
8) Continue this process until several cuvettes have been matched together.

II. Calculation of Factor and Target O. D.

A. Measure optical density (O.D.) on the standard and unknown solutions as follows:

1) Warm up the instrument for at least 10 minutes.
2) Adjust the wavelength control to the 540 mu setting.
3) Carefully wipe the lower third of a calibrated 12 x 75 mm** cuvette containing at least 2.0 ml of the reference solution (cyanmethemoglobin reagent only). This solution is called the "reagent blank." Properly position the cuvette in the cuvette well.
4) Adjust the Galvanometer Coarse and Fine controls until the galvanometer index reads 100% on the black transmittance scale (or zero on the red O.D. scale). Many spectrophotometers employ an additional control called the "zero adjust", which is used to set the needle on 0% T when the cuvette well is empty and covered.
5) Remove the reagent blank and replace it with a matched cuvette containing the sample solution. Again, wipe the cuvette clean and properly position it as before.
6) Read the O. D. of the sample on the optical density scale.

*Cuvettes already calibrated to ± 0.25% T may be purchased from several laboratory supply houses and from Hycel.

**13 x 100 mm cuvettes (minimum volume 3.0 ml) are used in certain instruments.

B. Preparation of Cyanmethemoglobin Reagent (Hycel)

1) Dilute one vial of reagent in two liters of distilled water (instead of one liter as indicated on the label). This helps to overcome the "resistant cell phenomenon."
2) Store this reagent in a brown screw-capped polyethylene bottle or in the dark, and at room temperature. (Do not use rubber or cork stoppers unless they are covered with parafilm, as there is a chemical reaction between the cyanide and these materials which results in contamination of the reagent.
3) Discard the reagent if it becomes cloudy or if a precipitate forms after prolonged use.

C. Preparation of Cyanmethemoglobin Standard Curve (hycel) and Calculation of the Target O.D.'s

1) The following concentrations of standard solutions should be used to calculate a factor: 80, 60, 40, 20, and 0 mg% cyanmethemoglobin.
2) Prepare these concentrations by diluting the 80.0 mg% cmg (undiluted) standard as follows:

Cyanmet. concentration (mg%)	Tube #1 80	Tube #2 60	Tube #3 40	Tube #4 20	Tube #5 0(blank)
Volume of 80 mg% standard (ml)	4	3	2	1	0
Volume of cyanmet. reagent (ml)	0	1	2	3	4

3) Wrap cork stoppers in parafilm and plug each tube. The standards will remain stable for several months provided they are kept refrigerated and free from contamination.
4) Read the O. D. of the standards at 540 mu. Be sure to use only matched and clean cuvettes.
5) The O. D. readings are used to calculate a factor which will be used to convert the O. D. of unknown samples to mg% cyanmethemoglobin. This factor then enables calculation of the **target O. D.** which is needed to prepare standardized RBC suspensions.
   Calculate the factor for converting O. D. to mg% cmg by dividing the sum of the concentrations of the standards by the sum of the O. D. readings of the standards. For example:

Conc. of standards (mg% cmg)	O. $D._{540}$ readings of standards
80.0	0.460
60.0	0.350
40.0	0.240
20.0	0.120
0.0	0.000
200.0	1.170

$$\text{Factor} = \frac{200.0 \text{ mg\% cmg}}{1.170 \text{ O.D.}} = 170.94 \text{ mg\% cmg/O.D.}$$

   This Factor may be used without change so long as the same instrument is employed and has not been moved or repaired. The reliability of the instrument should be checked before each subsequent use by reading the O. D. of the 40 mg% cmg dilution and comparing it with the previous 40 mg% cmg values.
6) Using the factor you have computed for your spectrophotometer, fill in column 3 of Table 1, Column 3 is the **target O.D.** on **your** spectrophotometer for each cell suspension you will use in your work.

Target O.D. (Table 1, column 3) = $\frac{\text{mg\% cmg (Table 1, col. 2)}}{\text{Factor}}$

Examples (using the factor of 170.94 computed in step C5):

2.8% Sheep cells: $\frac{35.035 \text{ mg\% cmg}}{170.94 \text{ mg\% cmg/O.D.}}$ = 0.205 target O.D.

0.4% Mammalian cells: $\frac{5.005 \text{ mg\% cmg}}{170.94 \text{ mg\% cmg/O.D.}}$ = 0.0293 target O.D.

0.25% Goose cells: $\frac{3.009 \text{ mg\% cmg}}{170.94 \text{ mg\% cmg/O.D.}}$ = 0.0176 target O.D.

0.50% Chicken cells: $\frac{5.059 \text{ mg\% cmg}}{170.94 \text{ mg\% cmg/O.D.}}$ = 0.0296 target O.D.

These target O.D.'s as stated above, will be used for accurately diluting your 4% suspensions to any lesser concentration.

TABLE 1

TARGET VALUES FOR SEROLOGICAL TESTS EMPLOYING STANDARDIZED RED BLOOD CELL SUSPENSIONS

1 Desired % Susp.		2 Target Mg% Cmg (all spectro.'s)	3 Target O.D. for Your Spectrophotometer	4 Test
Mammalian	3.0	37.537		
	2.8	35.035		All CF's (sheep)
	2.5	31.26		Toxoplasma IHA & Mycoplasma IHA (sheep)
	2.0	25.025		Paul-Bunnell heterophile aby test (sheep)
				Ox Cell Hemolysin Test (cattle)
	0.4	5.005		Adenovirus HA-HI (monkey, rat)
				Measles virus HA-HI (monkey)
				Reovirus HA-HI (human)
				Enterovirus HA-HI (human)
				Myxovirus HA-HI (human, guinea pig) (pig)
				Murine viruses HA-HI (human, guinea pig), sheep, mouse)
Goose	0.25	3.009		Arbovirus HA-HI
Chicken — Baby Chick	0.50	5.059		Poxvirus HA-HI (chicken)
				Myxovirus HA-HI (chicken)
	0.25	2.746		Rubella virus HA-HI (2-day chick)

III. Preparation of Standardized Erythrocyte Suspensions

A. Obtain fresh cells (less than 4 days old for best HA results). Bleeding should be done using one volume of blood to four volumes of modified Alsever solution.

B. Wash the cells three times in PBS diluent in a 12- or 15-ml conical centrifuge tube. One-half to full speed in any clinical centrifuge is satisfactory. Carefully remove the buffy layer of white blood cells after each wash. Following the last wash, read the packed cell volume and dilute to approximately 4% suspension with PBS.

C. Mix well and carefully pipette (with a long-tipped Mohr measuring pipette) 1.0 ml of the "4%" suspension into a 25 ml volumetric flask.

D. QS to 25.0 ml with cyanmethemoglobin reagent. Mix well by inverting the flask at least ten times.

E. Allow to stand 20-60 minutes at room temperature.

F. Mix again. If avian cell solutions appear turbid, add a few milligrams (a "pinch") of saponin and mix. All avian cell solutions must be briefly centrifuged after lysis to remove cellular debris.

G. Warm up and check the spectrophotometer by reading the 40 mg% standard.

H. Pour enough of the hemolyzed sample into a calibrated, clean cuvette to nearly fill the tube.

I. Read the O.D. of the sample against the Reagent Blank at 540 mu, being careful to read the red (logarithmic) scale. This is the *Test O.D.*

J. Calculate the dilution needed to obtain the desired suspension:

$$\frac{\text{(O.D. of the test susp.) x (original vol. of test susp. } -1.0 \text{ ml)}}{\text{Target O.D. (Table 1, col. 3)}} = \text{Final vol. of desired suspension}$$

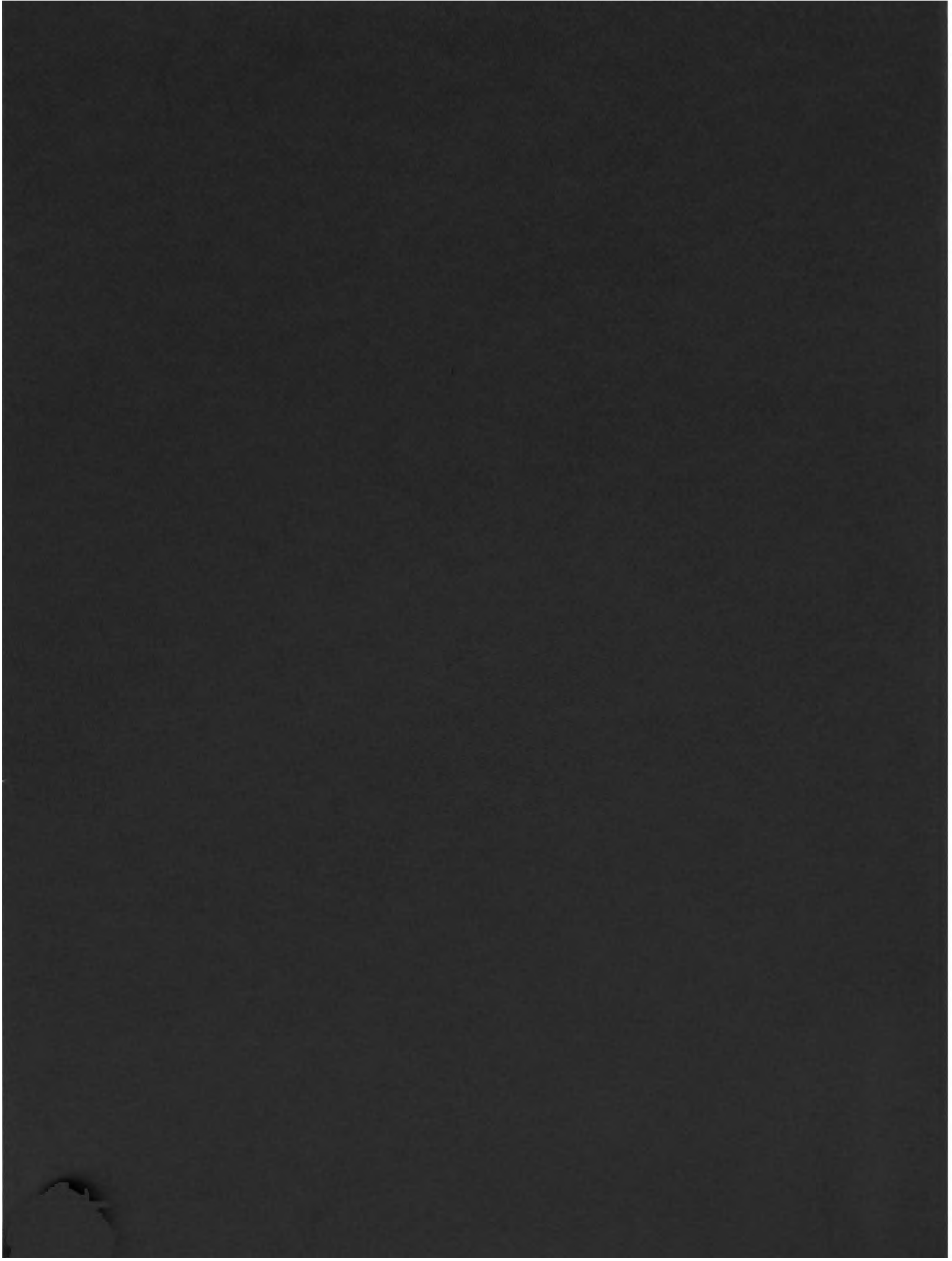
Example:

If you want a 0.5% suspension of chicken erythrocytes, and you start with 20 ml of a 4% suspension having a test O.D. of 0.358:

$$\frac{(0.358) \times 19 \text{ ml})}{0.0296} = 230 \text{ ml, final vol. of desired 0.5\% susp.}$$

The volume calculated in this formula is the total volume of the 0.5% suspension, i.e., 19 ml of the 4% suspension is diluted *to* a final volume of 230 ml to give 230 ml of a standardized 0.5% chicken RBC suspension. Be sure to mix the test suspension thoroughly but gently before preparing the diluted suspension.

It is not necessary to check the accuracy of the dilutions spectrophotometrically.



Microtitration Techniques

Many standard tests have been adapted to the use of microtitration techniques. The micro system permits savings in reagents as well as in performance time. Most of the procedures presented in this course will be done by microtechnique. It is essential that students become adept in the procedure both for the accomplishment of the course work and for accurate instruction of co-workers in the home laboratory.

Microtitration is a precisely engineered and highly reliable system for performing serological procedures. Many investigators have reported their work using the microtitration system. One of the earliest investigators and major contributors to the system was Takatsy. Since 1962, the microtitration system has gained wide acceptance in the United States and is now employed at the Center for Disease Control in the majority of laboratories performing routine sero-diagnostic work, and in many of the research laboratories.

The microtitration system is basically a volume reduction of the classical macrotitration (tube test). Volume decreases are made in a uniform manner so that proportionate volumes of the respective reagents are maintained. This reduction in volume will account for an eight-fold to twenty-fold savings in reagents and sera. Also, additional savings in time is accomplished by employing specially designed microtitration equipment engineered to deliver precisely calculated volumes.

Several manufacturers produce microtitration equipment. Basically the equipment consists of transparent plastic plates of reusable plastic or a vacuum formed disposable clear plastic, micro-diluters, and micro-droppers. Each plate contains 96 wells (or cups) and are available with either U or V shaped wells.

Dilutions are prepared by serial two-fold transfers of fluid with a microtitration diluter which picks up and transfers a precise volume. Several serial dilutions may be performed simultaneously as the diluters are designed with a special taper to give the same diluter spacing as the wells contained in the plastic plate. The diluters may be rinsed and flamed to cleanse them for reuse.

A pipette-dropper calibrated to deliver a pre-calibrated volume of fluid is used to deliver reagents such as buffers, antigens, and erythrocyte suspensions. The pipette-dropper is designed to be filled by mouth-suction or with a safety-suction bulb. The drops are easily controlled by a bulb or with the finger tip. After the addition of all the reagents to a microtitration test the plates may be stacked or taped to prevent evaporation during incubation. The disposable plastic plates float on the surface of a water bath, thus providing an incubation comparable to standard macro methods.

Tests are read in the same manner as macro tests, and the plates may be centrifuged with the use of special carriers to read tests which are interpreted by the percent lysis of the indicator cell system.

The microtitration system has proved its value in tests such as Complement Fixation, Hemagglutination, Hemagglutination-Inhibition, Antistreptolysin O Titrations, and others. It serves well in any procedure where dilutions are performed and erythrocytes are used as an indication of activity.

Practice Exercise

1. **Materials**
   a. **Reagents**
      1) Dye
      2) Buffer
      3) Distilled water
      4) 0.5% sodium hypochlorite
   b. **Equipment**
      1) Diluters, 0.025 and 0.05
      2) Pipette droppers, 0.025 and 0.05
      3) Microtitration plates
      4) Go-No-Go delivery testers, 0.025 and 0.05
      5) Plate holders
      6) Two beakers, 200 ml
      7) Bunsen burner
      8) Cotton swabs

2. **Methods**
   a. Procedures
      1) Handling of the microtitration diluters
         a) Flame the **coiled** wire diluters gently by passing very quickly through the coolest part of the flame; **do not heat to incandescence.**
         b) Heat the **tulip** and **acorn** diluters to incandescence.
         c) Cool to room temperature.
         d) Place diluter in beaker of distilled water to pre-wet. Put just enough water in the beaker to cover only the calibrated portion. **Do not get water on the shaft of the diluter.**
         e) Rotate the handle to fill the diluter.
         f) Test accuracy of the diluter delivery before making dilutions.
         g) Place Go-No-Go delivery tester on a smooth, nonabsorbent surface. Use 0.025 ml circles for testing 0.025 ml diluters and 0.05 circles for 0.05 ml diluters.
         h) Touch the filled diluter to the marked center of a circle on a testing blotter. The moist area must completely fill the area inside the circle.
         i) If area is not completely filled, continue to pre-wet and retest. If area is not filled after 4 times, gently reflame the diluter. **Cool before re-wetting.** If diluter still does not deliver satisfactorily, set aside. Retest the next time tests are set up.
         j) Place diluters in buffer used in test. Immerse only the calibrated portion.
         k) Check for accuracy as with the distilled water.
      2) Handling pipette droppers of each size.
         a) Place plastic plate in front of you.
         b) Draw distilled water up into the dropper. Wipe off tip of dropper with facial tissue.
         c) Holding the dropper perpendicular to the plate, practice placing drops in each well.
         d) If drop is not accurate, remove with a cotton swab (eraser).
         e) Practice placing the drops in the wells until accuracy is attained.

3) Making dilutions

a) Observe Pattern for Microtitration Practice (Page 14).
b) With a 0.025 ml dropper, place one drop of buffer into each well in a plastic plate, except the first well in each row.
c) With a disposable pipette, place 0.05 ml of dye solution (to represent a starting serum dilution) into the well in each row.
d) Place diluters in the first well of each row.
Use a separate diluter for each row of diluting to be prepared. Use as many diluters at a time as you can handle accurately and comfortably.
e) Rotate 4 seconds.
f) Remove from these wells and place them in the next well of the same row.
g) Rotate the loops 4 seconds.
h) Repeat this procedure until desired number of serial dilutions are prepared.
i) If diluters have been used in noninfectious material, check the accuracy of delivery immediately by touching to blotter. If the area within the circle is not filled, this dilution series has not been made accurately and must be repeated.
j) Place in distilled water.
k) If diluters are not to be used again, rotate, blot, flame, and store.
l) If diluters are to be used again, begin with step 1-d. The beaker of distilled water should be changed periodically during the test to insure proper rinsing and prewetting of the diluters.
m) Empty the contents of the practice plate into the sink, and repeat the practice procedure until you feel you are able to handle the equipment.

3. Precautions and Helpful Suggestions

a. Calibrated pipette droppers

1) Gently blot excess solution from the outside of the pipette dropper with a cleansing tissue. Do not wipe or rub the plastic barrel because this will generate static electricity which interferes with dropping the reagent into wells of the plate.

2) Discard the first two or three drops from the pipette dropper.

3) Hold the pipette dropper in a vertical position when dropping reagents. Holding it at an angle affects the size of the drops.

4) Clean the pipette dropper after each day's use by unscrewing the metal tip and rinsing the tip and barrel 8-10 times with hot tap water followed by 3 rinses in distilled water. Droppers used for cell suspension should be rinsed in cold water first.

b. Microdiluters

1) Do not touch the rims of wells with the microdiluters because some of the liquid may be lost.

2) Avoid spattering or creating bubbles as they can affect accuracy.

3) Clean the microdiluters in distilled water followed by heating the tips in a flame. Do not put handles in the flame.

4) Be sure to use the proper size Go-No-Go circle to check the diluter in use.

c. Go-No-Go Blotters

1) Blotters must be placed on a smooth, dry, nonabsorbent surface.

2) Never use the same test circle more than once.

4. References

Takatsy, G. 1955. The use of spiral loops in serological and virological micro methods. Acta Microbiol. Acad. Sci. Hung. *3*:191.

Sever, J. L. 1962. Application of a microtechnique to viral serological investigations. J. Immunol. *88*: 320.

Washing Microtitration Pipette Droppers

1. **Contaminated pipettes.**
   a. Soak for 30 minutes in 0.5% sodium hypochlorite.[1] Draw the fluid into the pipettes. Continue as for uncontaminated pipettes.
2. **Uncontaminated pipettes[2]**
   a. Rinse in running tap water ten times. Fill and empty completely each time.
   b. Place in Haemo-sol[3] solution for 30-60 minutes. Immerse the dropper completely. If necessary to speed up the washing procedure, empty and refill the droppers several times during a 15-30 minutes soaking period.
   c. Rinse in running tap water. Fill and empty at least ten times.
   d. Rinse in distilled water. Fill and empty ten times.
   e. Place in a rack to dry.[4]

[1] Commercial chlorox is approximately 5% sodium hypochlorite, so a 1:10 dilution of this works well.

[2] Pipettes may be washed with the aid of a suction type washer, similar to the Clay-Adams washer used for serological blood pipettes.

[3] 1 oz. Haemo-sol per four liters of tap water at 52°C.

[4] Pipettes may be placed in a 37°C dry incubator to speed the drying process.

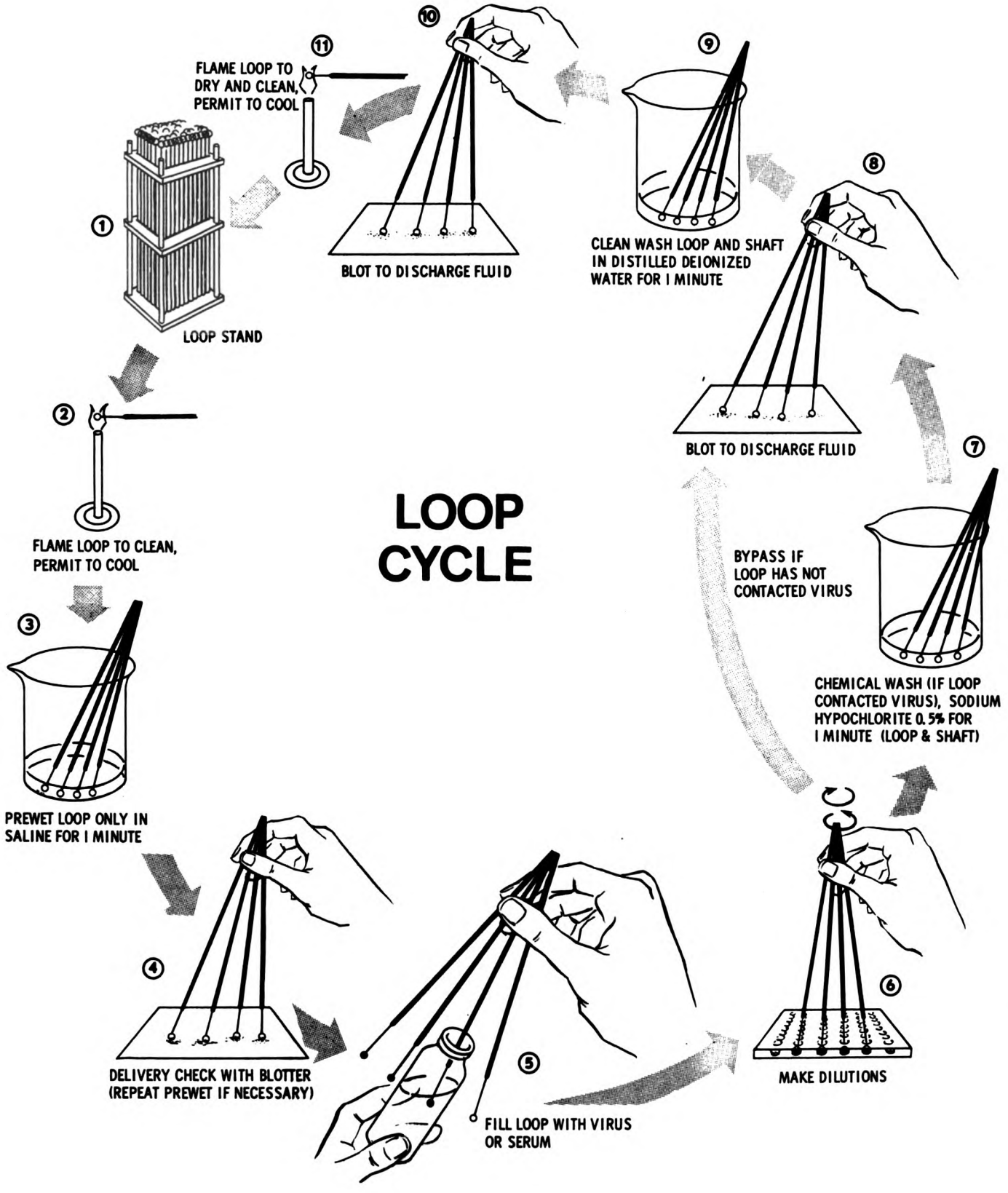
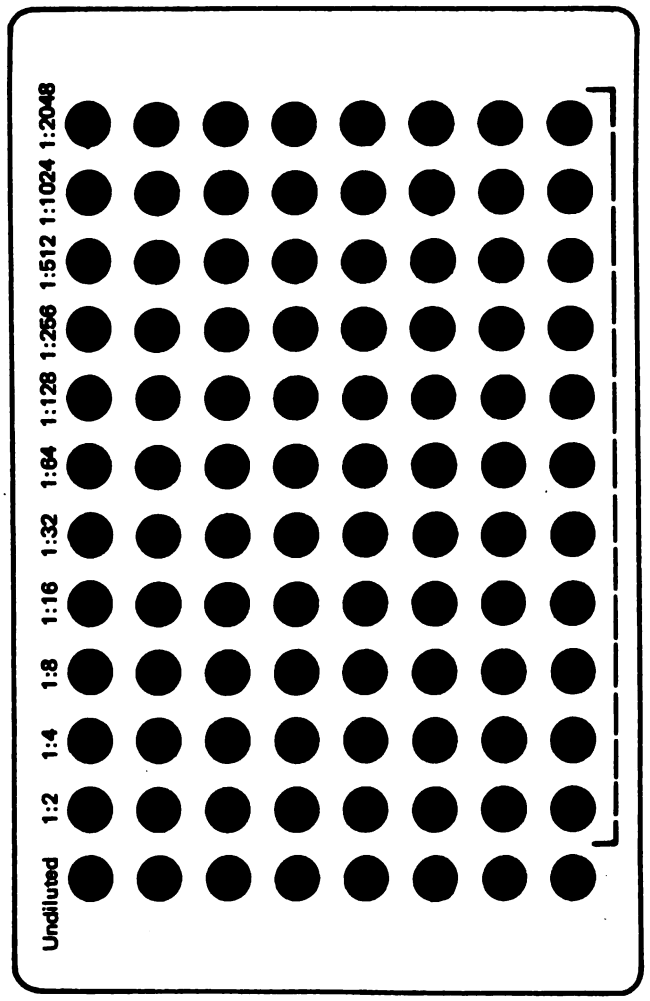

LOOP CYCLE
1
LOOP STAND
2
FLAME LOOP TO CLEAN, PERMIT TO COOL
3
PREWET LOOP ONLY IN SALINE FOR 1 MINUTE
4
DELIVERY CHECK WITH BLOTTER (REPEAT PREWET IF NECESSARY)
5
FILL LOOP WITH VIRUS OR SERUM
6
MAKE DILUTIONS
7
CHEMICAL WASH (IF LOOP CONTACTED VIRUS), SODIUM HYPOCHLORITE 0.5% FOR 1 MINUTE (LOOP & SHAFT)
BYPASS IF LOOP HAS NOT CONTACTED VIRUS
8
BLOT TO DISCHARGE FLUID
9
CLEAN WASH LOOP AND SHAFT IN DISTILLED DEIONIZED WATER FOR 1 MINUTE
10
BLOT TO DISCHARGE FLUID
11
FLAME LOOP TO DRY AND CLEAN, PERMIT TO COOL

PLATE PATTERN FOR MICROTITRATION PRACTICE



KEY

● TEST WELLS

○ EMPTY WELLS

Febrile Agglutination Tests

The examination of patients' sera for agglutinins against *Salmonella typhosa* in the classic Widal procedure is one of the oldest approaches used in a serologic laboratory. The test has been modified for identification of other agglutinins, among them other *Salmonella* species, *Brucella* species, *Francisella tularensis*, and *Proteus* species. The value of this test for *S. typhosa* agglutinins declined as (1) general use of vaccine increased, (2) incidence of typhoid fever sharply decreased, and (3) ever-increasing numbers of antigenically related serotypes of *Salmonella* were recognized. With the development of specific serologic tests for identification of rickettsial antibodies, the use of the agglutination procedure with *Proteus* antigens also has declined. Studies of agglutinins against *Brucella* and *F. tularensis* are still in use in most public health laboratories.

1. **Principles**
   a. Particulate antigens are aggregated into clumps in the presence of homologous antiserum as a result of the combination of antibody in the serum with multivalent antigen on the particle surface.
   b. The amount of antibody present is determined by combining serial dilutions of the serum with a constant amount of a standardized suspension of organisms.
   c. The highest final dilution of serum yeilding 50% clearing of the supernate is considered the endpoint of the reactivity.

2. **Tube Febrile Agglutination Test**
   a. **Materials**
      1) Test tubes, 13 x 100 mm
      2) Test tube racks
      3) Pipettes, serological, 1 ml, 2 ml, 5 ml
      4) Flasks, 125 ml, 250 ml
      5) 37°C water bath
   b. **Reagents**
      1) 0.85% NaCl
      2) phenolized saline: 0.85% NaCl with 0.5% phenol (ACS, Reagent Grade)
      3) formalinized saline: 0.85% NaCl with 0.5% formalin (ACS, Reagent Grade)
      4) Febrile antigens - properly diluted
         a) *Brucella abortus*
         b) *Francisella tularensis*
      5) Sera
         a) *Brucella abortus* antiserum
            1. Strong Positive (1:320 to 1:1280)
            2. Weak Positive (1:40 to 1:160)
         b) *Francisella tularensis* antiserum
            1. Strong Positive (1:320 to 1:1280)
            2. Weak Positive (1:40 to 1:160)
         c) Negative serum (<1:20)
         d) Patients' sera

c. **Procedure**

1) Set up racks as follows:

Rack #1 – Label for *B. abortus* antigen. Set up 4 rows of tubes, 9 tubes per row. Label row 1 - *B. abortus* (Strong) positive serum; row 2 - *B. abortus* (Weak) positive serum; row 3 - Negative serum; row 4 - Patient's serum. Include two additional tubes in each run; one designated antigen control and one designated reading standard.

Rack #2 – Label for *F. tularensis* antigen. Set up 4 rows of tubes, 9 tubes per row. Label row 1 - *F. tularensis* (Strong) positive serum; row 2 - *F. tularensis* (Weak) positive; row 3 - Negative serum; row 4 - Patient's serum. Include two additional tubes in each run; one designated antigen control and one designated reading standard.

2) Preparation of serum dilutions

a) Using a 2 ml pipette, place 0.9 ml saline in the first tube of each series of tubes and place 0.5 ml of saline in each of the remaining tubes in each row and the antigen control tube. Place 0.75ml of saline in the reading standard tube.

b) With a 1 ml pipette, place 0.1 ml of serum into tube 1 in the appropriate rows. This gives an initial dilution of 1:10 in the first tube.

c) With a 1 ml pipette, mix the contents in tube 1 and transfer 0.5 ml to tube 2. Using the same pipette, mix the contents of tube 2 and again transfer 0.5 ml to tube 3. Continue through the end of the dilution series. Discard 0.5 ml from the last tube. Repeat this with each row of serum.

3) Addition of antigen

a) Add 0.5 ml of diluted antigen suspension to tubes 1 through 9 of each row of the serum dilution series and to the antigen control tube. Final serum dilutions are now 1:20 to 1:5120.

b) Add 0.25 ml of diluted antigen suspension to the reading standard tube.

4) Shake the racks 10 times to thoroughly mix tube contents and incubate them in a 37°C water bath for the following times:

Rack #	Antigen	Time
1	*B. abortus*	44 to 48 hours
2	*F. tularensis*	18 to 24 hours

5) Reading

a) Remove tubes from water bath taking care not to disturb the sedimented antigen.

b) Tubes may be read using any good light source, but best results are obtained with a fluorescent desk lamp and a black background.

c) Examine all tubes individually before shaking and compare density of supernate with the "reading standard" tube.

d) Following the initial reading, shake individual tubes lightly and re-examine, noting degree of agglutination and again, density of unagglutinated organisms in suspension.

6) Serum titer

The titer of the serum is designated as the highest dilution of serum showing at least 50% clearing of supernate (2 plus reading).

7) Reporting

Report final dilution of serum in endpoint tube; e.g., Titer = 1:320.

Key for Reading Febrile Agglutination Test

4 plus (++++)	:	Complete agglutination and sedimentation; supernatant fluid clear.
3 plus (+++)	:	75% of organisms agglutinated; supernate slightly cloudy.
2 plus (++)	:	50% of organisms agglutinated; supernate 50% less dense than antigen control tube.
1 plus (+)	:	25% of organisms agglutinated; supernate slightly less dense than antigen control tube.
Negative (–)	:	No organisms agglutinated; suspension like antigen control tube.

3. **Antigen Standardization**

   a. ***Brucella abortus***

      1) Antigen should be shown to have the same reactivity as antigen prepared by the National Animal Disease Laboratory (NADL), Ames, Iowa.
      Note: The NADL antigen is accepted as the **standard antigen** because it is prepared in accordance with WHO recommendations and it is standardized against an International or National Reference Serum.

      2) Dilute antigen with phenolized saline according to producer's instructions.

      3) Discard any unused diluted antigen.

   b. ***Francisella tularensis***

      Use antigen that is pre-market tested by the Center for Disease Control. Dilute with formalinized saline according to producer's instructions.

4. **Precautions and Helpful Suggestions**

   a. Include antigen, control reading standard, and quantitative positive and negative serum controls in each test run.

   b. Read all control tubes first. If the controls are not as expected, discard the tests. The antigen control and reading standard must contain no agglutinated particles; the strong and weak positive controls must read at expected titer plus or minus one tube; and the negative control must show complete nonreactivity.

   c. Only antigens that have been standardized for their reactivity and specificity are to be used.

   d. Make measurements and dilutions carefully and accurately.

   e. Check temperature of water baths before and during incubation.

   f. Follow recommended time and temperature of incubation exactly.

5. **Interpretation**

   a. ***Brucella***

      1) Cross reactions with *Brucella* antigens are ordinarily found only in sera from tularemia patients or from persons vaccinated against cholera.

      2) Acute brucellosis

         a) Little or no titer may develop during the first ten days of illness.
         b) Acute and convalescent phase serum specimens, taken 2-4 weeks apart and tested in the same test run, are essential for demonstration of active infection. A **fourfold rise in titer is significant.**
         c) The titer may decline after the sixth week, or may persist as high as 1:160 for years after apparent clinical recovery. Therefore, a titer of 1:60 or above is indicative of *Brucella* infection at some time but not necessarily of current or recent infection.

3) Chronic brucellosis

There is no definite criterion whereby the significance of an agglutination test titer may be judged. No diagnostic question is likely to be raised about persons yielding high titers (titers higher than 1:160) and clinical findings that are compatible with modern knowledge of brucellosis while showing no symptoms of tularemia. Even though a titer of 1:20 is obtained, infection with *Brucella* cannot be ruled out on the basis of agglutination tests alone. In fact, some individuals although infected do not develop agglutinins.

4) Persons who have recovered from brucellosis may have Brucella antibodies restimulated non-specifically by any subsequent febrile illness. In such instances agglutination titers may rise to 1:160 or sometimes higher in a few days and drop to negative or 1:20 within 10 days. Such reactions confuse test interpretation.

b. *Francisella tularensis*

1) Acute and convalescent phase serum specimens, taken 2-4 weeks apart and tested in the same test run, are essential for demonstration of active infection. **A fourfold rise in titer is significant.**

2) If a single specimen only is available, a titer of **1:160 or above** is indicative of a *Francisella* infection at some time, but not necessarily of current or recent infection. Agglutinins decline slowly but may be detectable for life.

3) Vaccination will give the same agglutination picture as that seen in the natural infection.

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Indirect Hemagglutination (IHA) Test for Mycoplasma Pneumoniae

Mycoplasma pneumoniae was established in the early 1960's as the etiologic agent of primary atypical pneumonia associated with the production of cold hemagglutinins. Laboratory procedures as aids to diagnosis include isolation of the organism and/or serologic studies. Isolation of *Mycoplasma pneumoniae* from patients may require from three to four weeks for detection and identification. A more rapid diagnosis of mycoplasma infection can usually be made by performing serologic tests on paired sera. With the development of methods for reagent preservation the indirect hemagglutination (IHA) test has become of practical use. It is of particular value in smaller laboratories receiving only a few specimens daily.

1. **Principles**
   a. A stable *Mycoplasma pneumoniae* antigen, prepared by concentration and sonication of the organisms, will coat the tannic acid-treated surface of an essentially inert carrier, the sheep red blood cell.
   b. When specific antibody is added to the treated cells, visible clumping occurs.
   c. The amount of *Mycoplasma pneumoniae* antibody present is determined by combining serial dilutions of the serum with a constant amount of a standardized suspension of red blood cells sensitized with the specific antigen.
   d. The reciprocal of the highest initial dilution yielding a clearly positive agglutination of sensitized red blood cells is considered the endpoint of the reactivity.

2. **Materials**
   a. **Reagents**
      1) Acute and convalescent sera
      2) *Mycoplasma pneumoniae* antigen
      3) Tannic Acid, diluted 1:1000, 0.5 ml
      4) Sheep erythrocytes, 2.5% in PBS, pH 7.2
      5) Horse serum diluent (NHS) (1:150 dilution of horse serum in PBS)
      6) PBS, pH 6.4
      7) PBS, pH 7.2
   b. **Equipment**
      1) Water bath - 37°C
      2) Water bath - 56°C
      3) Centrifuge with equipment to handle 15 ml centrifuge tubes
      4) Centrifuge tubes, 15 ml: 3
      5) Erlenmeyer flasks, 200 ml: 4
      6) Microtitration equipment
         a) Droppers
         b) Testers
         c) U plates
         d) Cotton swabs

3. **Methods**

a. **Preparation of reagents**

pH 6.4 Phosphate Buffered Saline (PBS)

0.15M NaCl	100 ml	
0.15M Na_2HPO_4	32.2 ml	**Check pH**
0.15M KH_2PO_4	67.7 ml	

pH 7.2 Phosphate Buffered Saline (PBS)

0.15M NaCl	100 ml	
0.15M Na_2HPO_4	76.1 ml	**Check pH**
0.15M KH_2PO_4	23.9 ml	

Normal Horse Serum Diluent (NHS)

Normal horse serum is inactivated at 56° for 30 minutes and diluted 1:5 in pH 7.2 PBS. This treated serum may be stored frozen. It is diluted 1:30 with pH 7.2 PBS to give a final dilution of 1:150 (0.7%) for use in the test.

2.5% Sheep Red Blood Cells (SRBC)

1) Wash 3 ml sheep cells 3 times in pH 7.2 PBS for 5 minutes at 1700 rpm (600 x g). Bring the volume to 10 ml with pH 7.2 PBS for each washing.
2) Pack the cells for 10 minutes and make a 2.5% suspension (39 volumes pH 7.2 PBS to 1 volume packed cells).

50% Sheep Red Blood Cells

1) Wash as above.
2) Pack the cells for 10 minutes and make a 50% suspension (50 volumes of pH 7.2 PBS to 50 volumes of packed cells).

b. **Tanning of Standardized cells**

1) Prepare fresh tannic acid for each experiment.
   a) Weigh tannic acid in mg amount.
   b) For each 1 mg of tannic acid, add 1 ml pH 7.2 PBS to yield a final dilution of 1:1000.
   c) Dilute the 1:1000 stock solution 1:20 to obtain the 1:20,000 dilution for use in test:

      0.5 ml 1:1000 plus 9.5 ml **pH 7.2 PBS**

2) Mix 5 ml of 2.5% cells with 5 ml of the 1:20,000 tannic acid in *pH 7.2 PBS* in a 15 ml centrifuge tube.
3) Incubate tube in a 37°C water bath for 15 minutes.
4) Centrifuge for 5 minutes, remove supernatant fluid, suspend cells in 10 ml of *pH 7.2 PBS* and again centrifuge for 5 minutes.
5) Remove supernatant fluid and resuspend the cells to a 2.5% suspension by adding *pH 6.4 PBS* to a final volume of 5 ml. Mix the contents of the tube well.

c. **Sensitization of cells**

1) Prepare the optimal dilution of antigen in pH 6.4 PBS. (The optimal dilution as determined by block titrations against homologous immune *M. pneumoniae* serum is the lowest dilution of antigen yielding the highest specific immune serum titer.

2) Using two 15 ml graduated centrifuge tubes, pipette the following:

Mix well by carefully pipetting fluid up and down several times.

Tube 1: 1.0 ml 2.5% tanned cells
1.0 ml optimal antigen dilution

Tube 2 (control tube): 1.0 ml 2.5% tanned cells
1.0 ml pH 6.4 PBS

3) Incubate both tubes for 15 minutes at 37°C.

4) Centrifuge, remove supernatant and wash cells 2 times in the NHS diluent. Bring the volume to 2 ml for each washing.

5) After the second wash, remove the supernatant carefully and adjust the cells to 1% by bringing the volume to 2.5 ml with NHS diluent.

6) The 1.0% suspensions of tanned, sensitized cells (tube 1) and tanned unsensitized cells (tube 2) are now ready for use in the test.

d. Performance of the test

1) Inactivate sera at 56°C for 30 minutes.

2) Dilute 1:10 in NHS diluent (0.1 ml + 0.9 NHSD = 1:10).

3) Mark off the U plates so that there are 2 rows of 9 wells for each serum. In addition, mark 2 wells for the cell controls (one sensitized and one unsensitized).

4) With a pipette dropper, add 0.05 ml normal horse serum diluent to all wells in which serum dilutions will be made except for the first well in each row.

5) Add 0.05 ml normal horse serum diluent to the 2 wells which will serve as the cell controls.

6) With 0.5 ml pipettes, place 0.1 ml of the treated, 1:10 dilution of serum into the first wells of the 2 rows marked for each serum.

7) Make dilutions of serum with 0.05 ml loops which have been pretested for accurate delivery.

8) Pipette 0.025 ml of tanned, sensitized red cells into the first row of each set of serum, and into the well marked for the sensitized cell control.

9) Pipette 0.025 ml of tanned, unsensitized red cells into the second row of each serum and into the well marked for the unsensitized cell control. Shake plates on vibrator for 60 seconds.

10) Cover the plates and incubate at room temperature for 4 hours.

11) The reciprocal of the highest initial dilution yielding a clearly positive agglutination of sensitized red cells is considered an endpoint.

12) If agglutination of control cells in the second row is equal to that of the sensitized cells, the serum must be sorbed and the test repeated:

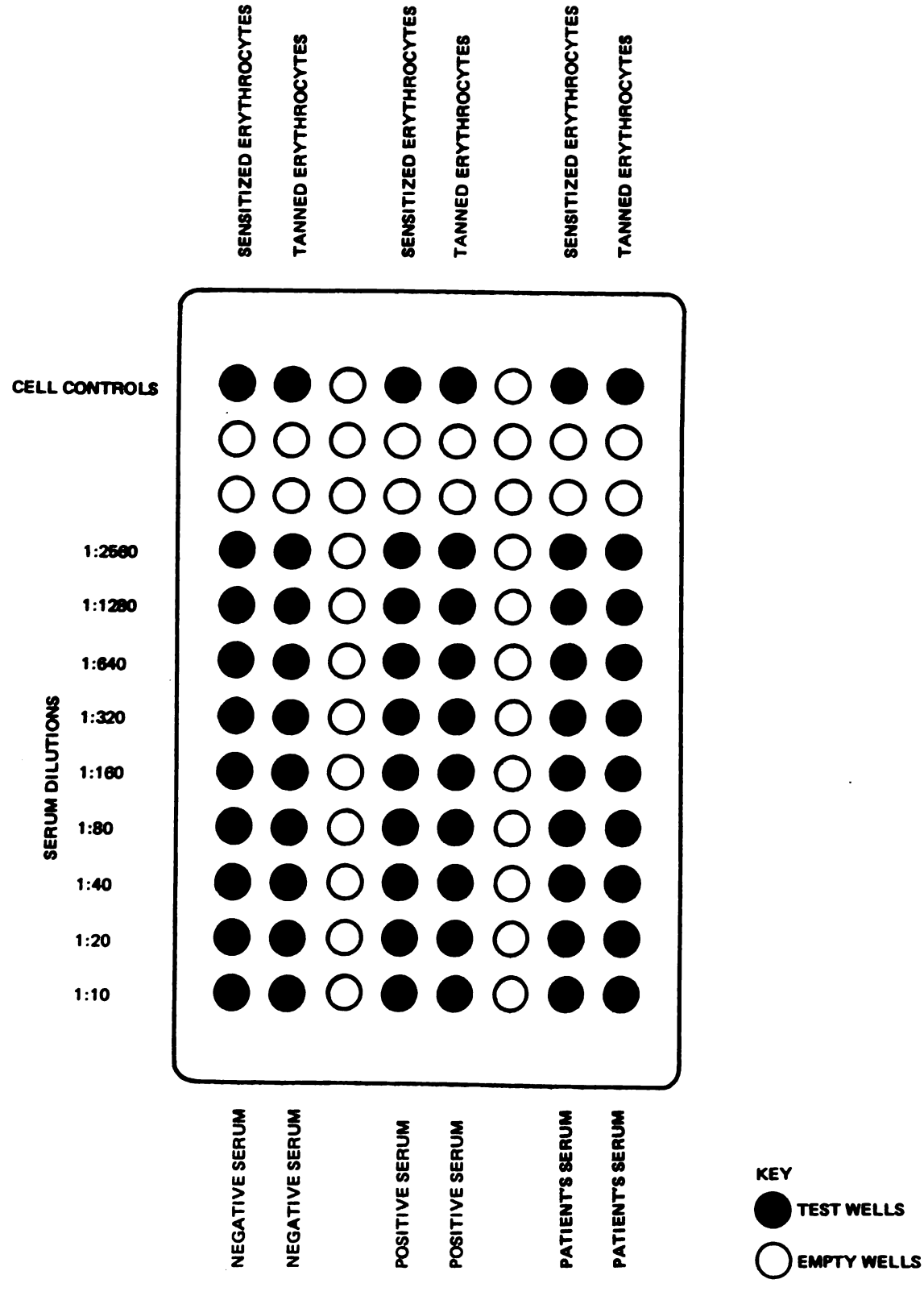
a) Inactivate serum and dilute 1:10 in NHS diluent.

b) Sorb two or more times with 0.1 ml of 50% SRBC per ml of diluted serum for 30 minutes at 4°C.

c) Centrifuge at 1700 rpm for ten minutes.

d) Remove the supernate; this is a 1:10 dilution of treated serum.

PLATE PATTERN
MYCOPLASMA PNEUMONIAE IHA TEST



4. Precautions and Helpful Suggestions

a. If sorption of serum with 50% SRBC fails to remove nonspecific agglutination, serum must be sorbed (following the same method) with SRBC sensitized with horse serum.

b. Sera from children usually show nonspecific agglutination.

c. Sera from adults usually do not show nonspecific agglutination.

d. Tests may be read after overnight incubation at room temperature; the patterns are clearer and more easily interpreted. Titers usually are the same or show no more than one-tube difference when compared with those after the 4-hour-incubation period.

e. Store stock antigen at −20°C to prevent deterioration.

f. Glutaraldehyde-treated cells give a different pattern than nontreated cells.

5. Interpretation

Specific agglutination of *M. pneumoniae* sensitized RBC indicates the presence of *M. pneumoniae* antibodies. A fourfold or greater rise in titer with paired sera suggests current or recent infection.

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MYCOPLASMA PNEUMONIAE: ANTIGEN BLOCK TITRATION

1. **Materials**
   a. **Reagents**
      1) Specific immune serum, sorbed with sheep red blood cells.
      2) *Mycoplasma pneumoniae* antigen.
      3) Tannic acid (TA).
      4) Sheep red blood cells (SRBC) in Alsever's or 3.8% sodium citrate.
      5) Phosphate buffered saline (PBS), 6.4.
      6) Phosphate buffered saline (PBS), 7.2.
      7) Horse serum diluent (NHS), 1:150 dilution of horse serum in PBS, 7.2.
   b. **Equipment**
      1) Water bath, 37°C.
      2) Water bath, 56°C.
      3) Centrifuge equipment to handle 15 ml and 40 ml centrifuge tubes.
      4) Centrifuge tubes, 15 ml.
      5) Erlenmeyer flasks, 200 ml.
      6) Microtitration equipment.
      7) Rack which will hold 40 ml centrifuge tube.

2. **Methods**
   a. **Preparation of reagents**
      1) **pH 6.4 Phosphate Buffered Saline (PBS)**

0.15 M NaCl	100 ml	
0.15 M Na_2HPO_4	32.2 ml	**Check pH**
0.15 M KH_2PO_4	67.7 ml	

      2) **pH 7.2 Phosphate Buffered Saline (PBS 7.2)**

0.15 M NaCl	100 ml	
0.15 M Na_2HPO_4	76.1 ml	**Check pH**
0.15 M KH_2PO_4	23.9 ml	

      3) **Normal Horse Serum Diluent**

         Normal horse serum is inactivated at 56°C for 30 minutes and diluted 1:5 in PBS 7.2. This treated serum may be stored frozen. It is diluted 1:30 with PBS 7.2 to give a final dilution of 1:150 (0.7%) for use in the test.

      4) **Preparation of 2.5% Sheep Red Blood Cells (SRBC)**
         a) Wash 4 ml SRBC 3 times in PBS 7.2 *and centrifuge* for 5 minutes at 1700 rpm (600 x g).
         b) Pack the cells for 10 minutes and make a 2.5% suspension, (39 volumes PBS 7.2 to 1 volume packed cells).
      5) **50% Sheep Red Blood Cells (RBC)**
         a) Wash as above.
         b) Pack the cells for 10 minutes and make a 50% suspension in PBS 7.2.

6) **Tanning of Standardized Cells**

a) Prepare fresh tannic acid for each experiment.

(1) Weigh tannic acid in mg amounts.

(2) For each 1 mg of tannic acid, add 1 ml PBS 7.2 to yield a final dilution of 1:1000.

(3) Dilute the 1:1000 stock solution 1:20 to obtain the 1:20,000 dilution for use in test:

0.5 ml 1:1000 plus 9.5 ml pH 7.2 PBS

b) Mix 10 ml of 2.5% cells with 10 ml of the 1:20,000 tannic acid in PBS 7.2 in a 40 ml centrifuge tube.

c) Incubate tube in a 37°C water bath for 15 minutes.

d) Centrifuge for 5 minutes, remove supernatant fluid, suspend cells in 30 ml of PBS 7.2 and again centrifuge for 5 minutes.

e) Remove supernatant fluid and resuspend the cells to a 2.5% suspension by adding PBS 6.4 to a final volume of 10 ml. Mix the contents of the tube well.

7) **Preparation of Antigen Dilutions**

a) Place 15 ml graduated centrifuge tubes in the first 8 spaces of row 2 of a test tube rack and label from left to right: undiluted, 1:2, 1:4, 1:6, 1:8, 1:12, 1:16, and PBS, 6.4 (control tube).

b) Place 13 x 100 tubes in spaces 2 through 7 in row 1 and label each with the antigen dilution on the corresponding centrifuge tube in row 2.

c) Prepare the antigen dilutions in the 13 x 100 tubes using PBS 6.4 as the diluent.

Dilution	Antigen	PBS 6.4
1:2	1.2 ml, undiluted	1.2 ml
1:4	1.4 ml, 1:2	1.4 ml
1:6	1.0 ml, 1:4	0.5 ml
1:8	0.75 ml, 1:4	0.75 ml
1:12	0.5 ml, 1:6	0.5 ml
1:16	0.5 ml, 1:8	0.5 ml

8) **Sensitization of SRBC**

a) Place 0.5 ml of 2.5% tanned cells in each centrifuge tube marked for antigen.

b) Place 0.5 ml of 2.5% tanned cells in the centrifuge tube marked PBS, 6.4.

c) Add 0.5 ml of undiluted antigen and of each antigen dilution to the designated centrifuge tubes. Mix well by pipetting the mixture up and down several times.

d) Add 0.5 ml of PBS, 6.4 to the labeled control tube. Mix well.

e) Incubate the mixtures in the centrifuge tubes for 15 minutes at room temperature.

f) Centrifuge, remove supernatant, and wash cells 2 times in 2 ml of the normal horse serum diluent (NHS).

g) After the second wash, remove the supernatant carefully and adjust the cells to 1% by bringing the volume in each tube to 1.25 ml with NHS diluent.

h) The 1% suspensions of tanned, sensitized cells (sensitized with each antigen dilution) and the tanned, unsensitized cells (control tube) are now ready for use in the block titration.

9) **Serum Preparation**

a) Inactivate 0.2 ml of undiluted known specific immune serum (sheep red blood cells sorbed) at 56°C for 30 minutes. Cork the tube.

b) Dilute 1:10 in NHS diluent (add 1.8 ml NHS diluent to the undiluted, inactivated serum).

10) **Block Titration**

a) Mark 2 U plates according to the attached plate patterns. Note there are two rows for each antigen concentration.

b) With an 0.05 ml pipette dropper, add 0.05 ml NHS diluent to wells 2 through 9 and to well 12 of all rows of both plates.

c) With an 0.2 ml or 0.5 ml pipette, add 0.1 ml of the 1:10 dilution of inactivated serum to well 1 of each serum row.

d) Make dilutions of serum with 0.05 ml diluters which have been pretested for accuracy.

e) With an 0.025 ml dropper pipette, add 0.025 ml of tanned red blood cells sensitized with the proper antigen concentration into wells 1 through 9 and well 12 of row 1 and 2 of each designated antigen concentration set.

f) With an 0.025 ml dropper, add 0.025 ml of the tanned unsensitized red blood cells to the proper wells of the last 2 rows of the second plate. Shake plates on vibrator for 60 seconds.

g) Cover the plates (use another plate) and incubate at room temperature for 4 hours.

h) The optimal dilution of antigen is the **lowest** antigen dilution which shows the highest serum titer with no agglutination in the unsensitized well.

PLATE PATTERN (1)
MYCOPLASMA PNEUMONIAE: ANTIGEN BLOCK TITRATION

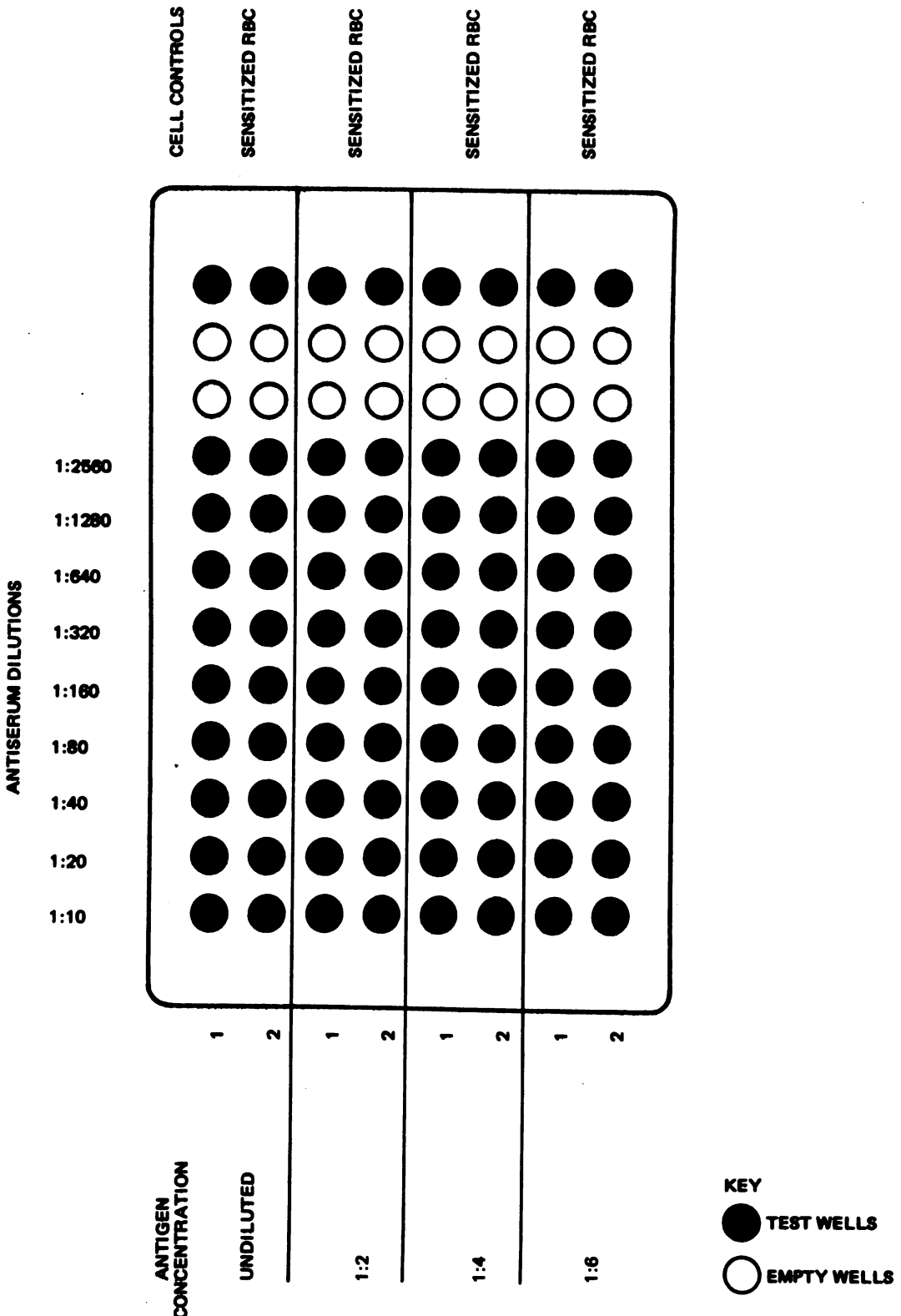
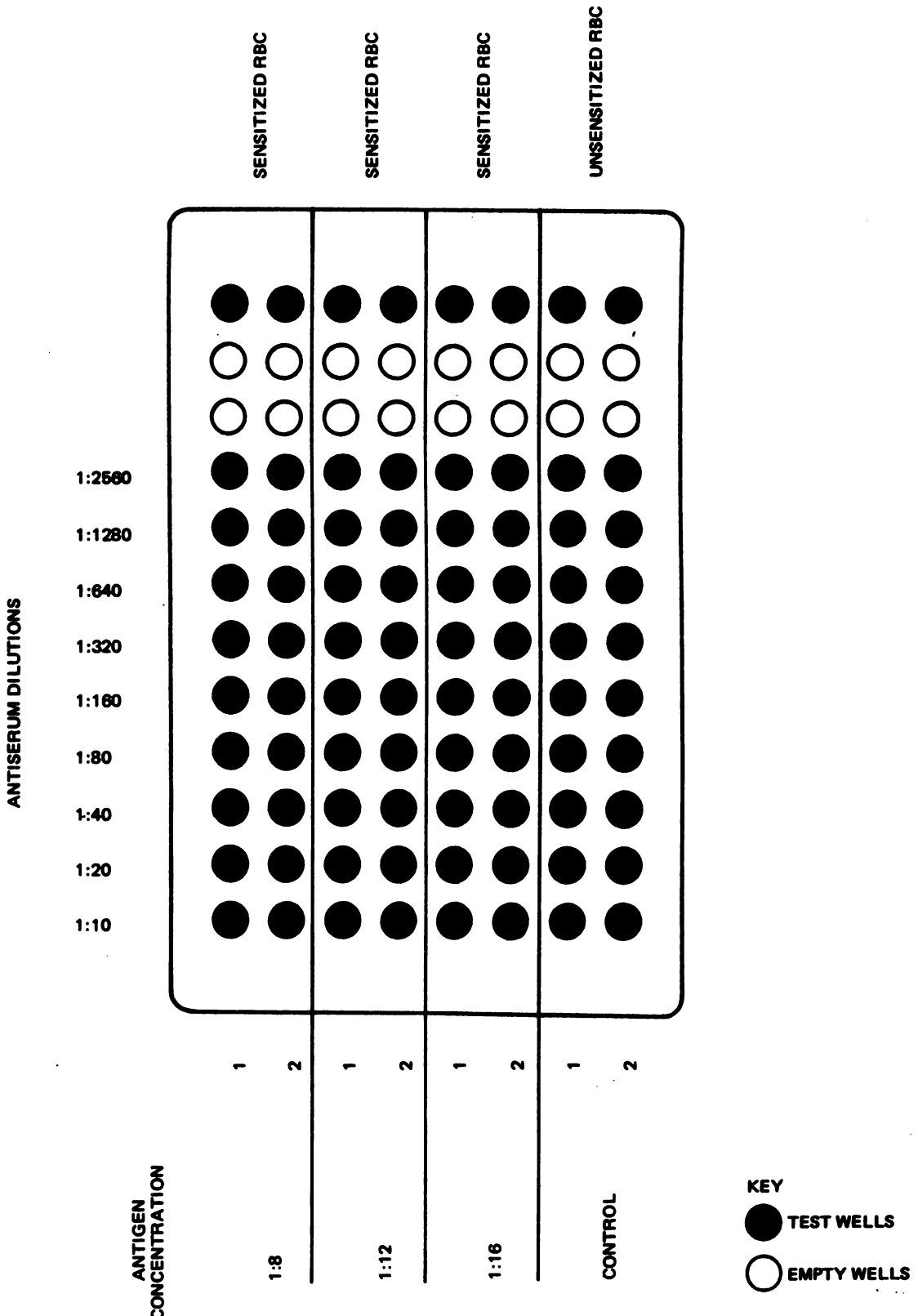


PLATE PATTERN (2)
MYCOPLASMA PNEUMONIAE: ANTIGEN BLOCK TITRATION (Cont'd)



Hemagglutination-Inhibition (HI) Test for Arboviruses

Arboviruses are a group of infectious agents biologically transmitted between susceptible vertebrate hosts by hematophagus arthropods. Frank disease may be characterized by a variety of syndromes, e.g., undifferentiated fever, dengue, hemorragic fever, hemorrhagic hepatitis, meningo-encephalitis, encephalitis. Predominately, infections are subclinical or atypical, recognized only through antibody studies.

1. **Principles**
   a. Arboviruses agglutinate the red blood cells of 24-hour-old chicks or of adult geese.
   b. Hemagglutination usually occurs within a pH range of 6.0 - 7.0, at temperatures between 4°C and 37°C, and at incubation periods of 30 to 60 minutes, depending upon the virus under study.
   c. Hemagglutination is inhibited specifically by antibodies in immune serum and nonspecifically by lipoprotein inhibitors in serum.
   d. Lipoprotein inhibitors routinely are removed by sorption with kaolin or bentonite (treated sera), or by extraction with acetone (treated sera).
   e. Arbovirus hemagglutination-inhibition activity is identified by incubating treated sera, serially diluted, with a constant amount of standardized antigen. The endpoint of HI activity is the reciprocal of the highest serum dilution showing complete inhibition of hemagglutionation.
2. **Materials**
   a. **Reagents**
      1) Sucrose-acetone antigens (SA), beta-propiolactone (BPL) treated, diluted 1:10 in 0.4% bovalbumin-borate saline, pH 9.0.
      2) White-goose erythrocytes, 1:24 dilution of stabilized, standardized cells in 0.2 M phosphate buffers.
      3) Phosphate buffers: 0.15 M NaCl - 0.2 M phosphate buffers at pH 5.75, 6.0, 6.2, 6.4, 6.6, 6.8, 7.0, 7.2, 7.4.
      4) Test sera, paired
      5) Control sera
      6) Kaolin, acid washed
      7) Acid-Citrate-Dextrose solution (ACD)
      8) Dextrose-Gelatin-Veronal Buffer (DGV)
      9) 0.4% bovalbumin-borate saline (BABS)
   b. **Equipment**
      1) Microtitration Equipment
         Droppers, 0.05, 0.025
         Diluters, 0.05, 0.025
         Diluter testers, 0.025, and 0.05
         U-plates
         Cellophane tape and dispenser
         Cotton swabs
      2) Mechanical vibrator
      3) Incubator or water bath at 37°C
      4) Water bath at 56°C
      5) Centrifuge with heads and carriers for 15 ml centrifuge tubes, blood sample tubes, and micro plates
      6) Spectrophotometer

7) Glassware

13 x 100 tubes (blood and serum)
Volumetric flasks, 100 ml and 1000 ml
Flasks for reagents: Buffers, cells, antigens
Conical 15 ml centrifuge tubes
Pipettes, sterile, plugged: 1.0 ml, 0.1 ml or 0.2 ml, 5.0 ml
Beakers, 250 ml: For buffer and distilled H_2O rinses for diluters

8) Other

Millipore filter apparatus and filters
Suction apparatus or dropping pipettes
Rubber bulb, 1.0 ml

3) Methods

a. Preparation of Reagents

1) Acid-Citrate-Dextrose (ACD) Solution

Dextrose	22.0 g
Sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$)	22.52 g
Citric Acid ($H_3C_6H_5O_7 \cdot 2H_2O$)	8.0 g
Distilled water qs, ad.	1,000 ml

Sterilize by filtration

2) Dextrose-Gelatin-Veronal (DGV)

5, 5-Diethylbarbituric acid (barbital)	0.58 g
Gelatin	0.60 g
Na 5, 5-Diethylbarbiturate (sodium barbital)	0.38 g
$CaCl_2$ (anhydrous)	0.02 g
$MgSO_4$	0.12 g
NaCl	8.50 g
Dextrose	10.00 g
Distilled water qs, ad.	1,000 ml

Dissolve the veronal and gelatin in 250 ml of distilled water by heating. Combine this solution with the other reagents. Sterilize by filtration.

3) 0.4% Bovalbumin-Borate-Saline (BABS), pH 9.0

1.5 M NaCl

NaCl	87.68 ml
Distilled water qs, ad.	1,000 ml

0.5 M Boric Acid

H_3BO_3	30.92 g
Hot distilled water (dissolve then cool)	700 ml
Distilled water qs, ad.	1,000 ml

1.0 N NaOH

This material may be purchased from commercial sources.

Borate Saline, pH 9.0

1.5 M NaCl	80 ml
0.5 M H_3BO_3	100 ml
1.0 N NaOH	24 ml
Distilled water qs, ad.	1,000 ml

Check the pH with a pH meter.

4% Bovalbumin (Fraction V), pH 9.0

Bovalbumin (Fraction V)	4 g
pH 9.0, Borate Saline	90 ml

Adjust to pH 9.0 with 2 N NaOH. Bring to 100 ml with pH 9.0 borate saline.

0.4% Bovalbumin-Borate-Saline (BABS), pH 9.0

pH 9.0, 4% bovalbumin	100 ml
pH 9.0 Borate saline	900 ml

Filter and store at 4°C.

4) Phosphate Buffers for Arbovirus Hemagglutination Tests

1.5 M NaCl

NaCl	87.68 g
Distilled water qs, ad.	1,000 ml

2.0 M Dibasic Sodium Phosphate

Na_2HPO_4 (anhydrous)	283.96 g
Distilled water qs, ad.	1,000 ml

2.0 M Monobasic Sodium Phosphate

$NaH_2PO_4H_2O$	276.02 g
Distilled water qs, ad.	1,000 ml

0.15 M NaCl – 0.2 M Na_2HPO_4

1.5 M NaCl	100 ml
2.0 M NaH_2PO_4	100 ml
Distilled water qs, ad.	1,000 ml

0.15 M NaCl – 0.2 M NaH_2PO_4

1.5 M NaCl	100 ml
2.0 M NaH_2PO_4	100 ml
Distilled water qs, ad.	1,000 ml

0.2 M Phosphate Buffers (For final dilution of goose erythrocytes)

Desired pH in Hemagglutination	0.15 M NaCl 0.2 M Na_2HPO_4	0.15 M NaCl 0.2 M NaH_2PO_4
pH 5.75	3.0 ml	97.0 ml
pH 6.0	12.5 ml	87.5 ml
pH 6.2	22.0 ml	78.0 ml
pH 6.4	32.0 ml	68.0 ml
pH 6.6	45.0 ml	55.0 ml
pH 6.8	55.0 ml	45.0 ml
pH 7.0	64.0 ml	36.0 ml
pH 7.2	72.0 ml	28.0 ml
pH 7.4	79.0 ml	21.0 ml

Check the pH of these buffer-mixtures by mixing equal parts of the 0.2 M phosphate buffer with 0.4% bovalbumin-borate saline, pH 9.0 This pH indicates the pH which is found in a test well containing 0.4% bovalbumin-borate saline, pH 9.0, and the diluted erythrocytes.

5) Preparation of Erythrocyte Suspension

a) Collect adult male white goose erythrocytes in acid-citrate dextrose (ACD) – 1.5 ml of ACD for each 8.5 ml of blood to be collected.

b) Centrifuge collected erythrocyte suspension at 1500 rpm for 15 minutes; discard supernatant.

c) Wash cells four times with dextrose-gelatin-veronal (DGV) solution. For each washing, the packed cells are resuspended in a sufficient volume of DGV (approximately three to four times the volume of cells) and centrifuged at 1500 rpm for 15 minutes.

d) After the final wash resuspend the cells in DGV. For each 1 ml of packed cells, add 10 ml of DGV. This cell suspension is stabilized overnight at 4°C to allow for any shrinkage of the cells.

e) Dilute one ml of the stabilized DGV-cell suspension with 39 ml of 0.9% NaCl. The optical density (OD) of this 1:40 suspension is measured in a Coleman Jr. Spectrophotometer at 490 mu, in cuvettes having a 10 mm internal diameter. The OD of the 1:40 cell suspension should be 0.450.

f) If the spectrophotometric reading is not 0.450, adjust the stabilized cells by adding or removing DGV according to the formula:

$$\text{Final Volume Desired} = \text{Initial Volume on Hand} \times \frac{\text{Observed OD}}{\text{Desired OD}}$$

g) The stabilized cells are ready for use in the HA or HI tests. For use in the microtitration procedure, make a 1:24 dilution of the stabilized and standardized goose erythrocyte suspension in each of the 0.2M phosphate buffers.

6) Preparation of Sera

a) Heat inactivation of sera is not necessary.

b) Treatment with Kaolin and goose erythrocytes

(1) **Reagents**

(a) Human or animal sera
(b) Kaolin, acid washed, 25% suspension in borate saline, pH 9.0

(2) **Procedures**

(a) Dilute each serum in a 15 ml conical centrifuge tube, 1:5 with borate saline, pH 9.0 (0.2 ml of serum plus 0.8 ml of borate saline).

(b) Add 1.0 ml of a 25% suspension of acid-washed kaolin to 1.0 ml of each 1:5 serum dilution.

(c) Incubate the serum-kaolin mixtures at room temperature for twenty minutes with frequent (2-3 times) shaking.

(d) Centrifuge the serum at 2500 rpm for 30 minutes (room temperature)

(e) The supernatant represents approximately a 1:10 dilution of the serum.

(f) Directly add 0.1 ml of washed and packed goose erythrocytes to the supernatant (serum).

(g) Place the serum in an ice bath for 20 minutes. Shake the mixture at 5 minute intervals.

(h) Following incubation, centrifuge again in the cold at 2500 rpm for 30 minutes and remove the supernatant. The supernatant (approximately 1:10 serum dilution) is ready for use in the HI test.

c) Other procedures for removal of nonspecific inhibitors of arbovirus hemagglutination are found in the **Appendix** of this section.

b. **Performance of Test**

1) Titration of antigens

a) Label microtiter "U" plates as indicated in the plate pattern (Hemagglutination Test, arbovirus antigen titration).

b) To each plate add 0.05 ml of bovalbumin-borate saline (BABS) 0.4%, pH 9.0 to the second through tenth and to the twelfth test wells in each row. The twelfth well is the cell control well.

c) Prepare a 1:10 dilution of antigen and keep overnight at 4°C prior to use in the test. This allows for dissociation of any aggregated antigen particles.

d) Add 0.1 ml of a 1:10 dilution of antigen to the first well of each row of one plate.

e) Prepare serial twofold dilutions (1:10 through 1:5120) of each arbovirus antigen with 0.05 ml diluters.

f) Place the plates on a mechanical shaker and add 0.05 ml of the goose erythrocyte suspension to the rows labeled with their respective pH's.

g) Seal the plates with cellophane tape and incubate in a 37°C water bath for 30 minutes.

h) Examine the plates after 30, 45, and 60 minutes of incubation. Record the hemagglutination as follows:

+ = complete agglutination

⊕= slight ring of non-agglutinated cells

± = trace of agglutinated cells around the bottom of non-agglutinated cells

0 = no agglutination

i) Determine the optimal pH and length of incubation for hemagglutination with each antigen. The highest dilution of antigen yielding complete agglutination is defined as one HA unit. The optimal pH is that pH yielding the highest HA titer and clearest HA pattern. The erythrocyte control should not show agglutination.

2) Hemagglutination-Inhibition (HI test for detection of viral antibody)

a) Label microtiter "U" plates as indicated in the plate pattern (Hemagglutination-Inhibition, Arbovirus). Label one for each antigen to be used.

b) Add 0.025 ml of BABS with an 0.025 ml pipette dropper to the three rows of ten wells (labeled SERUM DILUTIONS) omitting the first well in each row. Repeat this procedure for each of the two plates.

c) Add 0.025 ml of BABS to the wells labeled serum controls.

d) Add 0.05 ml of BABS to the erythrocyte control wells.

e) Add 0.05 ml of each of the treated sera to the first well of the indicated row of each plate. This is the 1:10 dilution.

f) Add 0.025 ml of each of the treated sera to the serum control well at the end of their respective rows.

g) Prepare serial twofold dilutions (1:10 through 1:5120) of the sera with 0.025 ml diluters.

h) Dilute the antigens in BABS to contain eight HA units per 0.025 ml*. Prepare a minimum of 2 ml. To determine the dilution of virus which contains eight HA units per 0.025 ml, divide the titer as determined in 0.05 ml volumes by sixteen.

*Antigen dilutions are prepared the day before use and kept overnight at 4°C prior to use in the test.

PLATE PATTERN
HEMAGGLUTINATION TEST
ARBOVIRUS ANTIGEN TITRATION

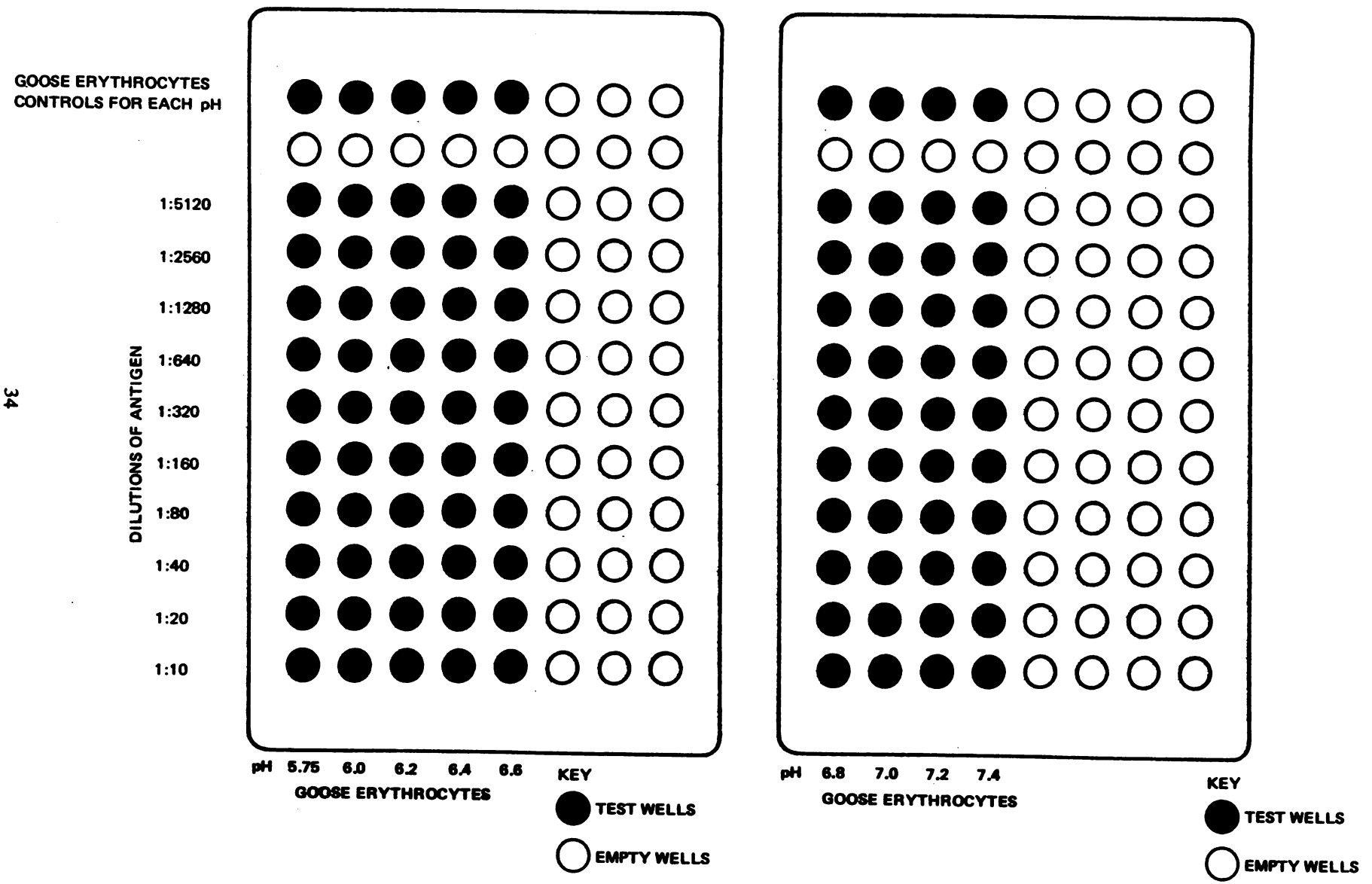


PLATE PATTERN
HEMAGGLUTINATION-INHIBITION TEST
FOR ARBOVIRUSES

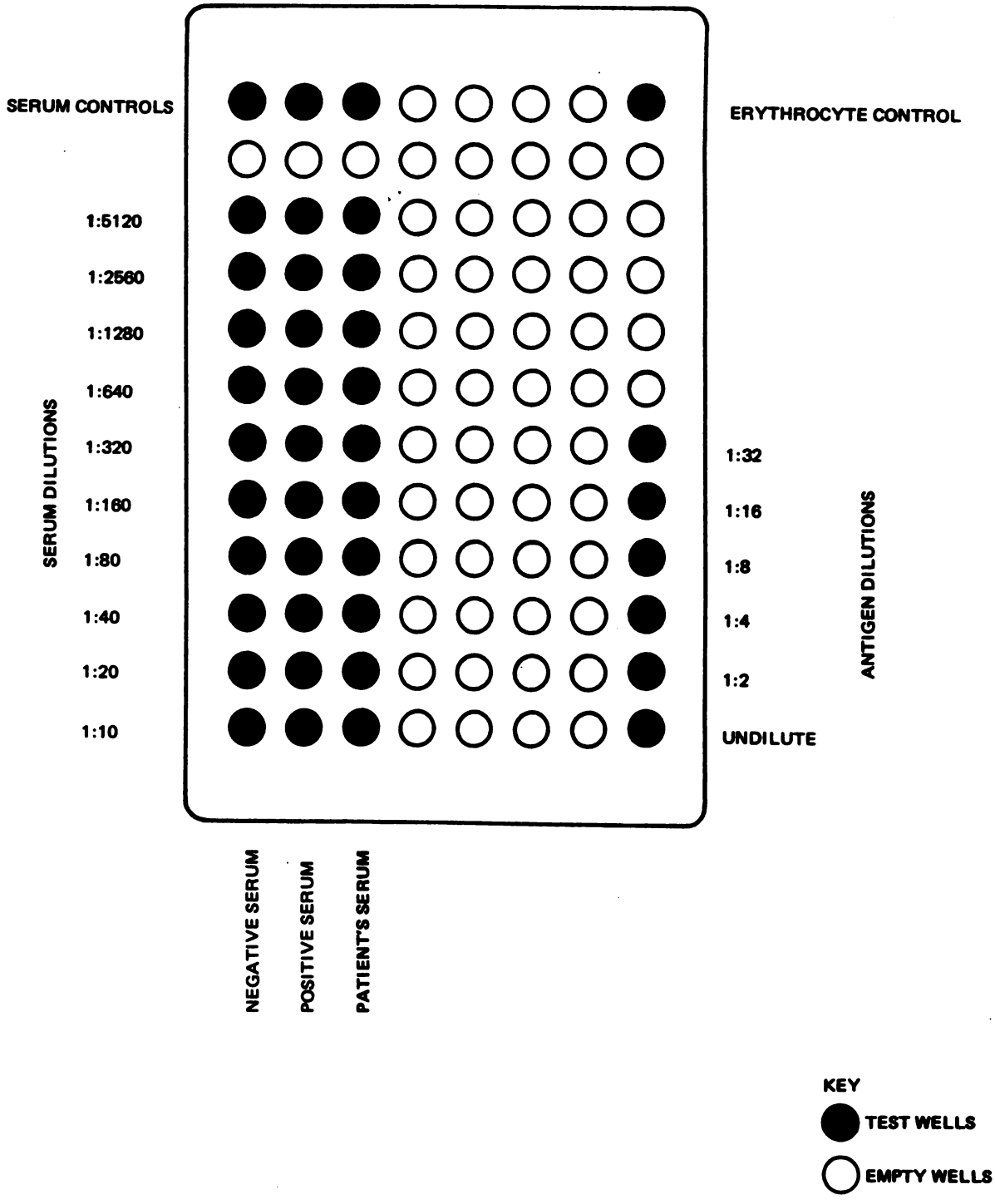


PLATE PATTERN
HEMAGGLUTINATION TEST
ARBOVIRUS ANTIGEN TITRATION

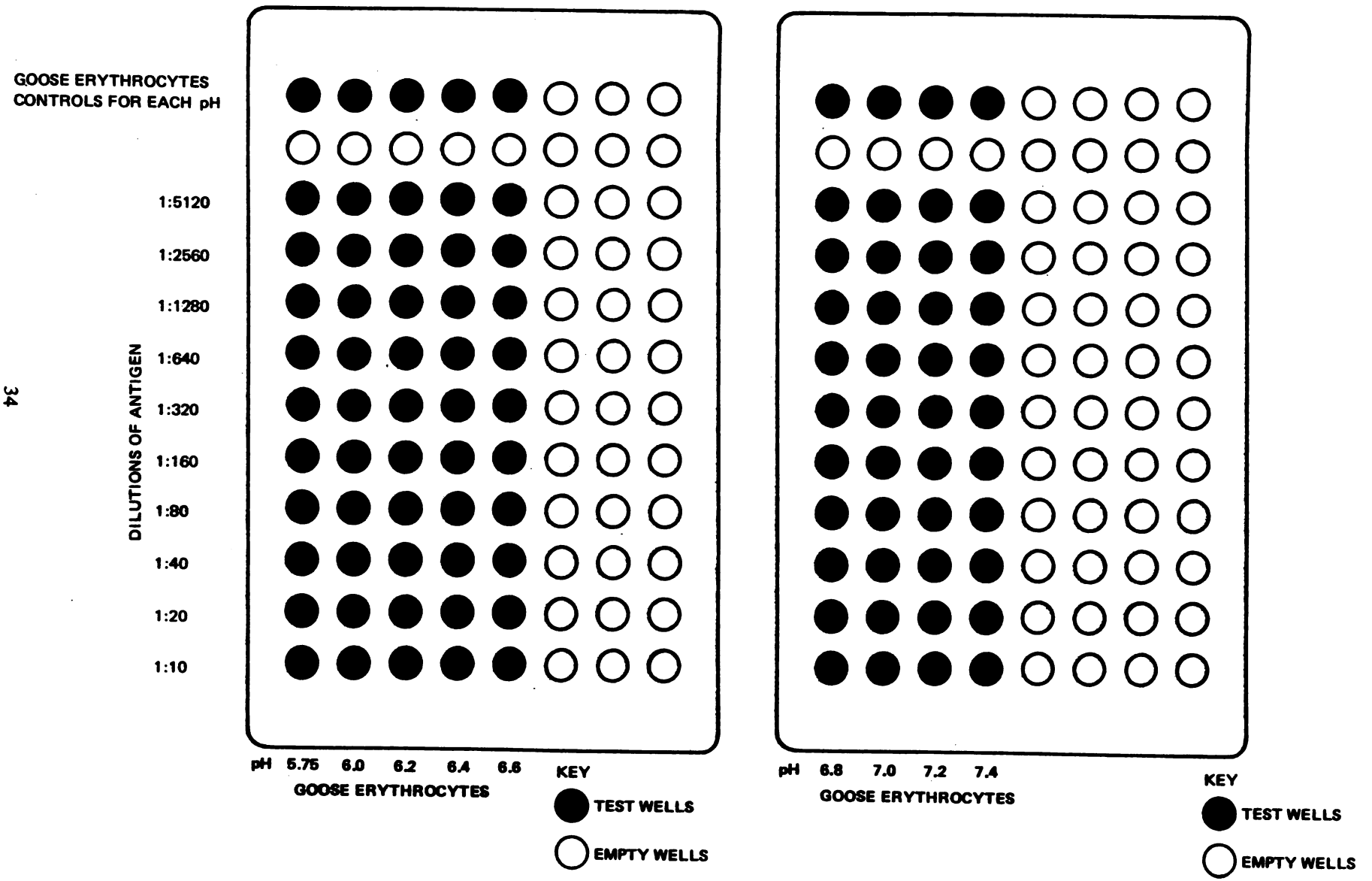


PLATE PATTERN
HEMAGGLUTINATION-INHIBITION TEST
FOR ARBOVIRUSES

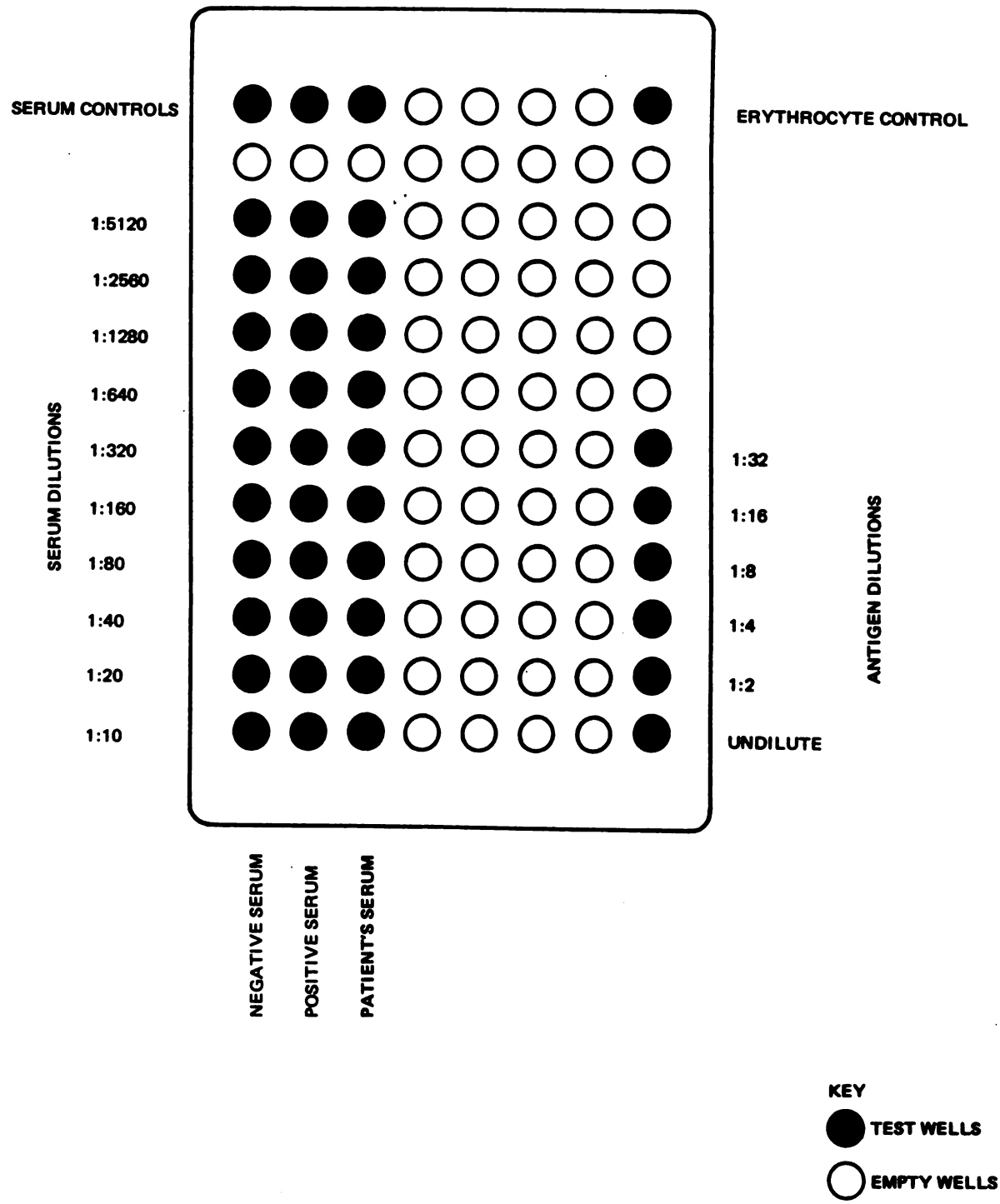


PLATE PATTERN
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ARBOVIRUS ANTIGEN TITRATION

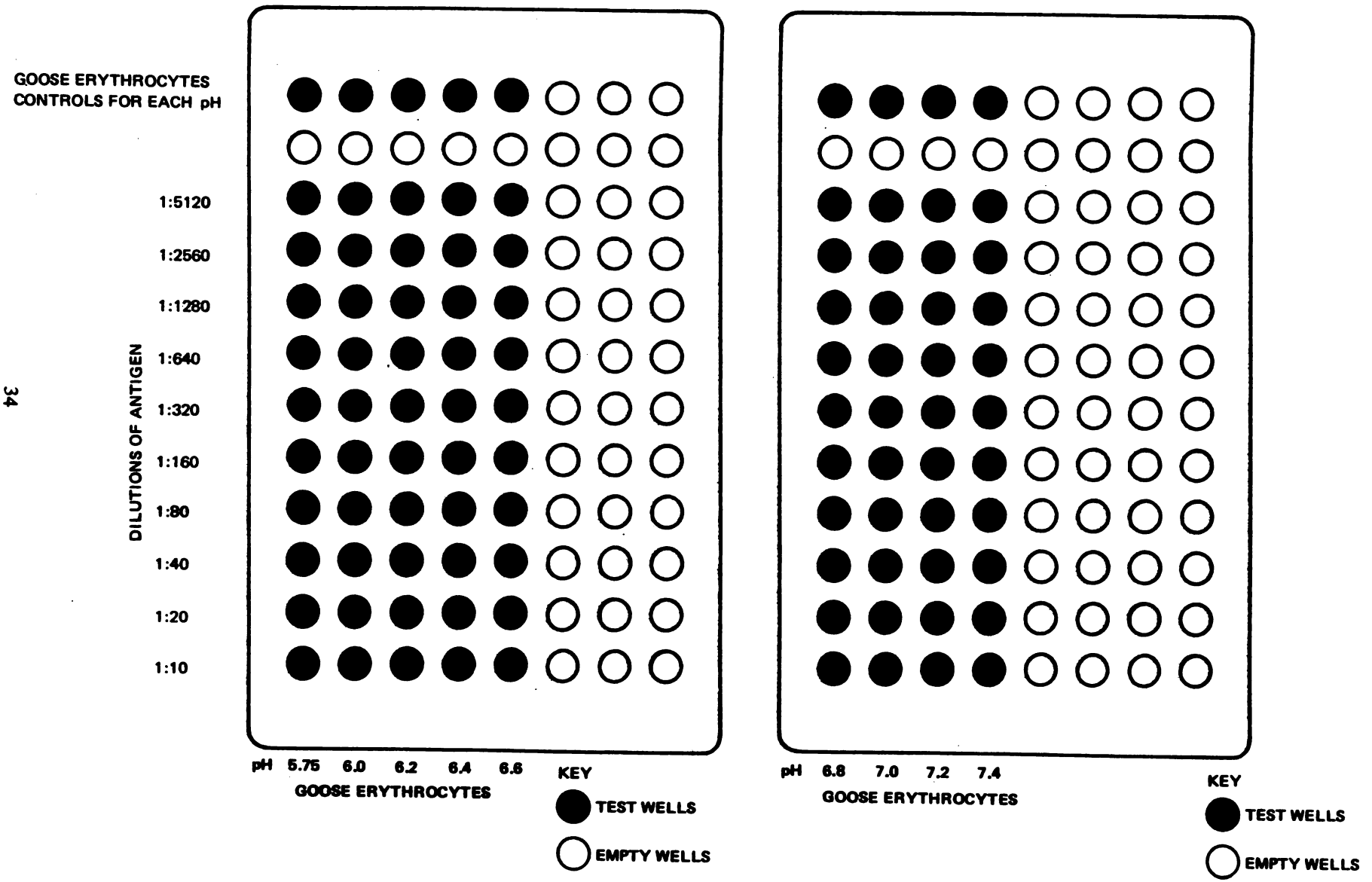

PLATE PATTERN
HEMAGGLUTINATION-INHIBITION TEST
FOR ARBOVIRUSES

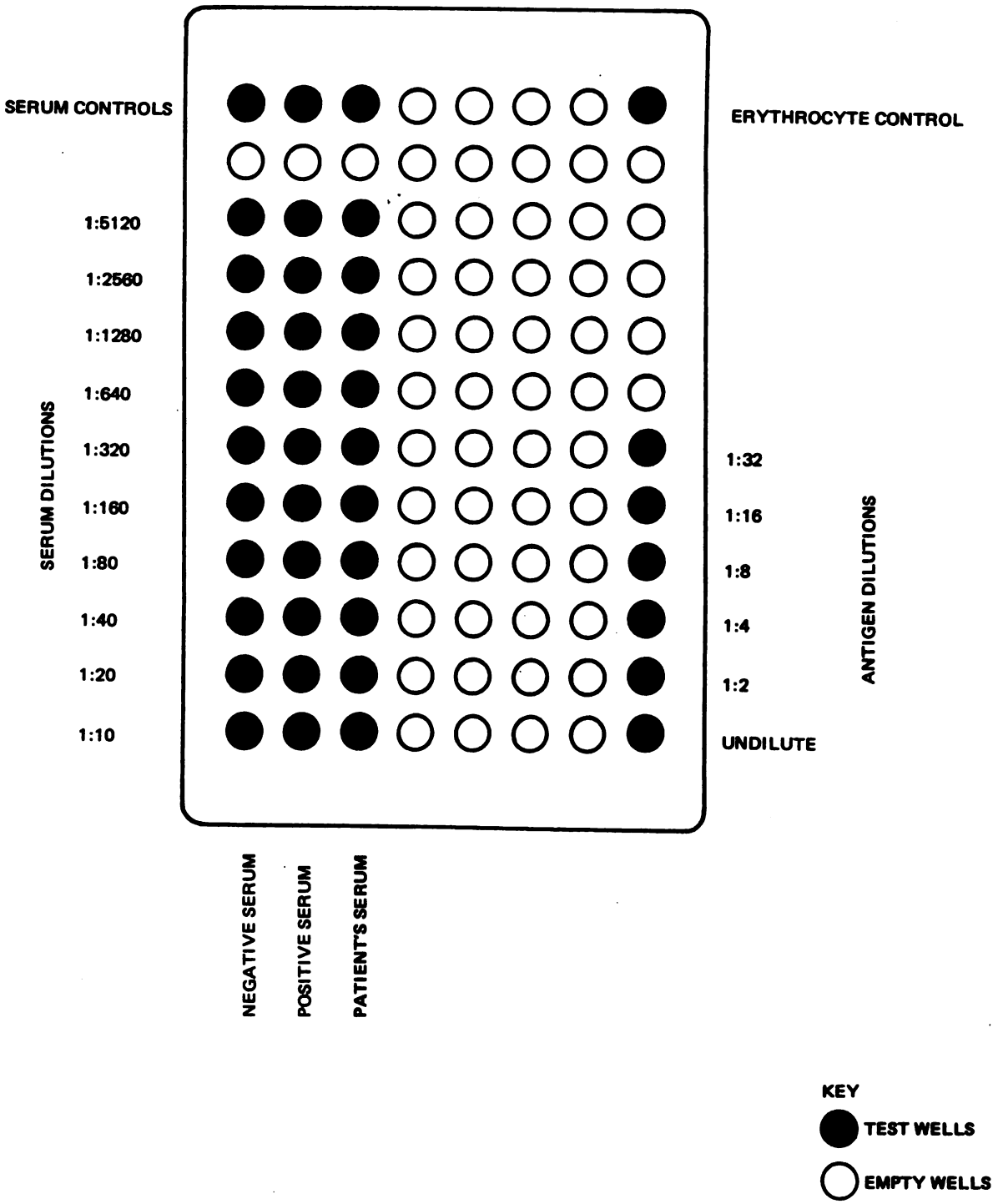

PLATE PATTERN
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ARBOVIRUS ANTIGEN TITRATION

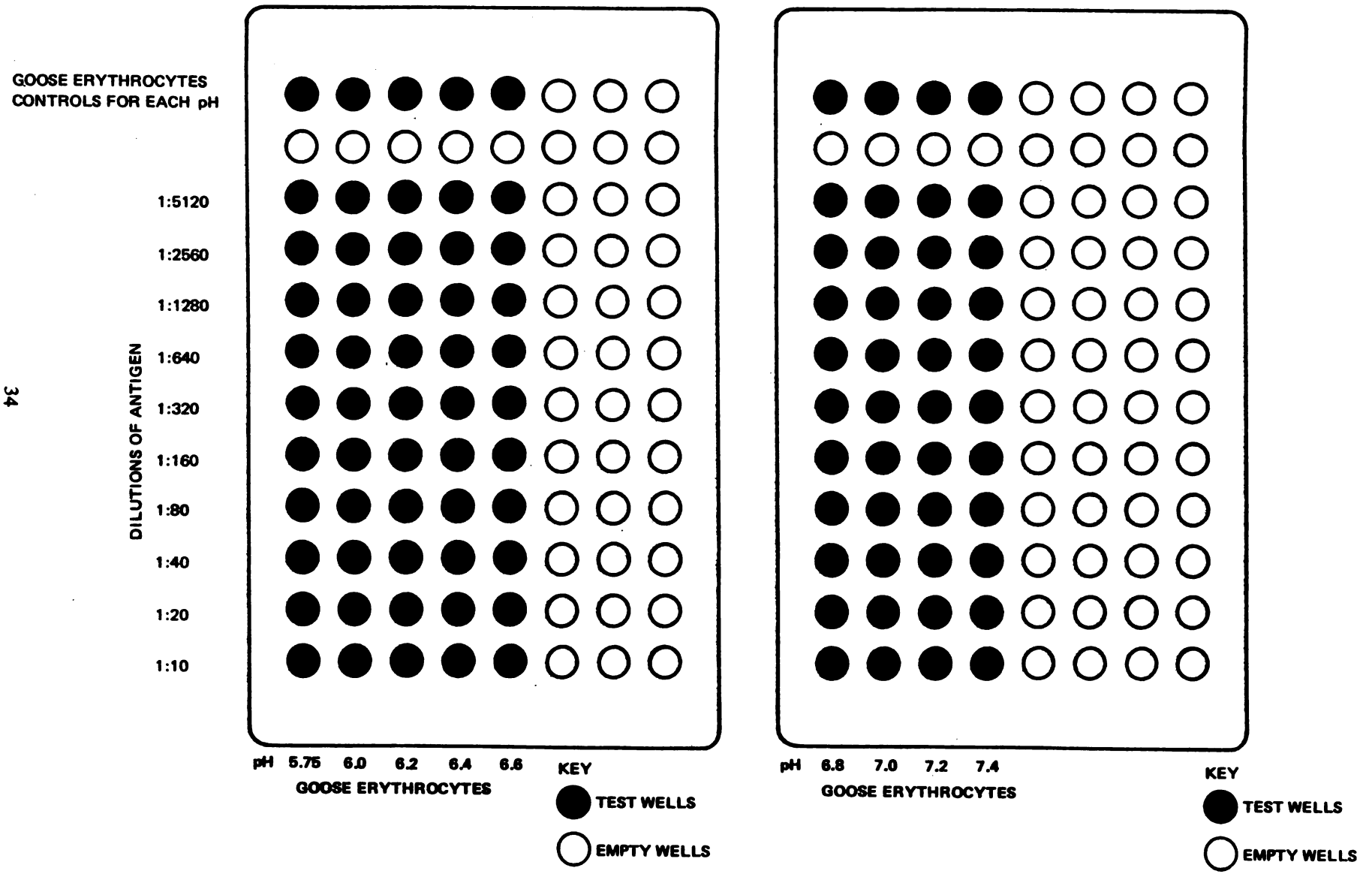


PLATE PATTERN
HEMAGGLUTINATION-INHIBITION TEST
FOR ARBOVIRUSES

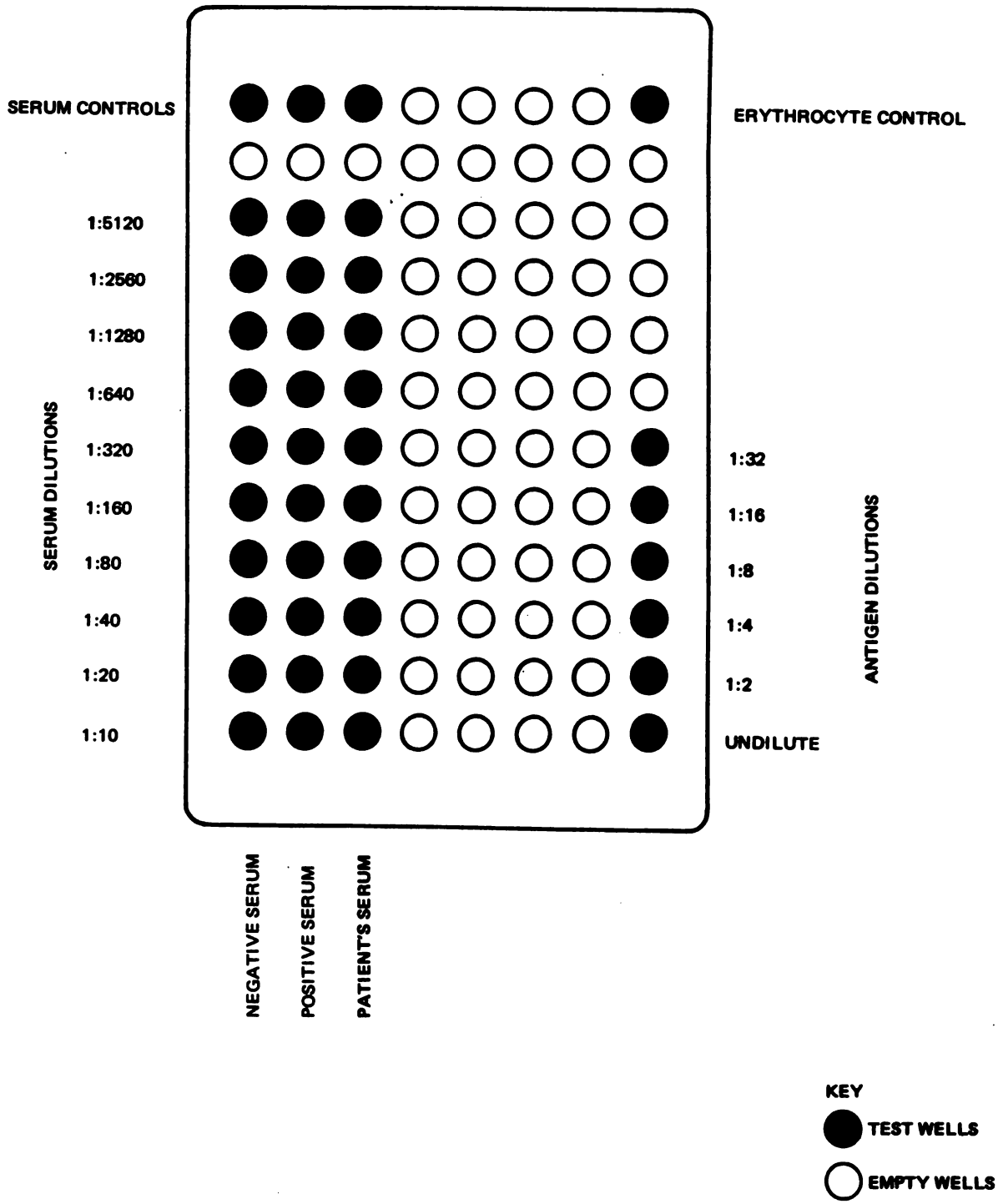


PLATE PATTERN
HEMAGGLUTINATION TEST
ARBOVIRUS ANTIGEN TITRATION

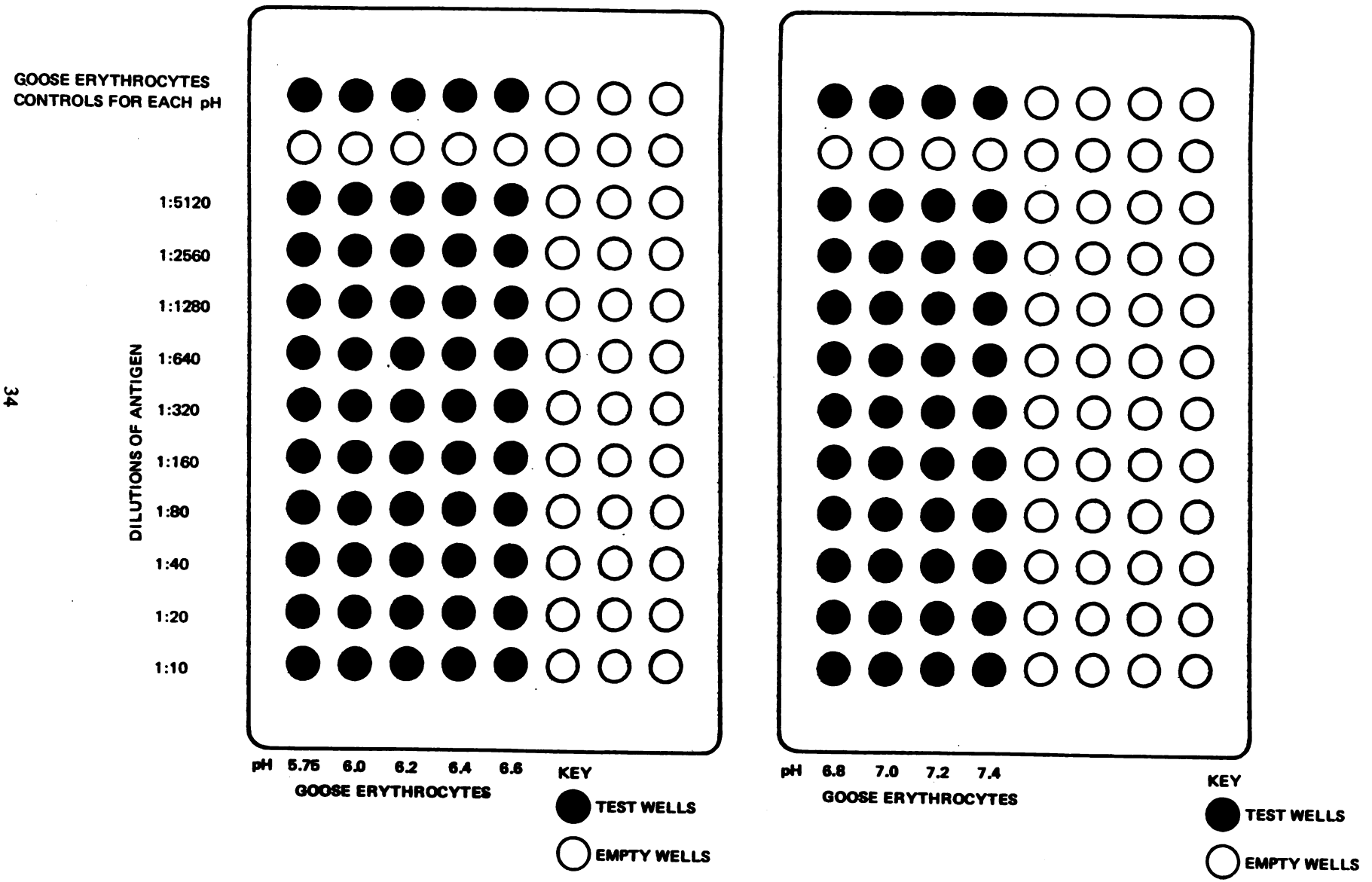


PLATE PATTERN
HEMAGGLUTINATION-INHIBITION TEST
FOR ARBOVIRUSES

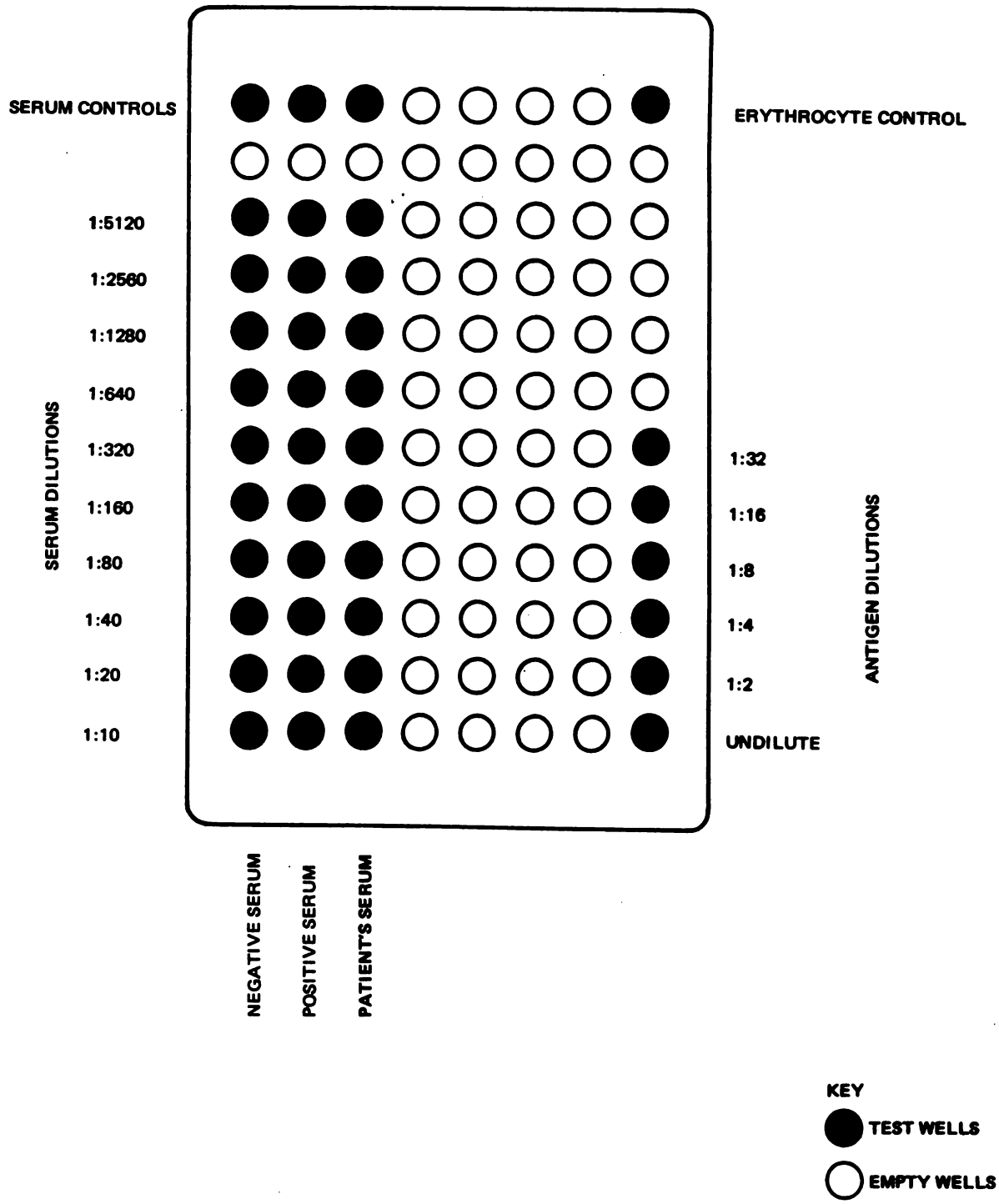


PLATE PATTERN
HEMAGGLUTINATION TEST
ARBOVIRUS ANTIGEN TITRATION

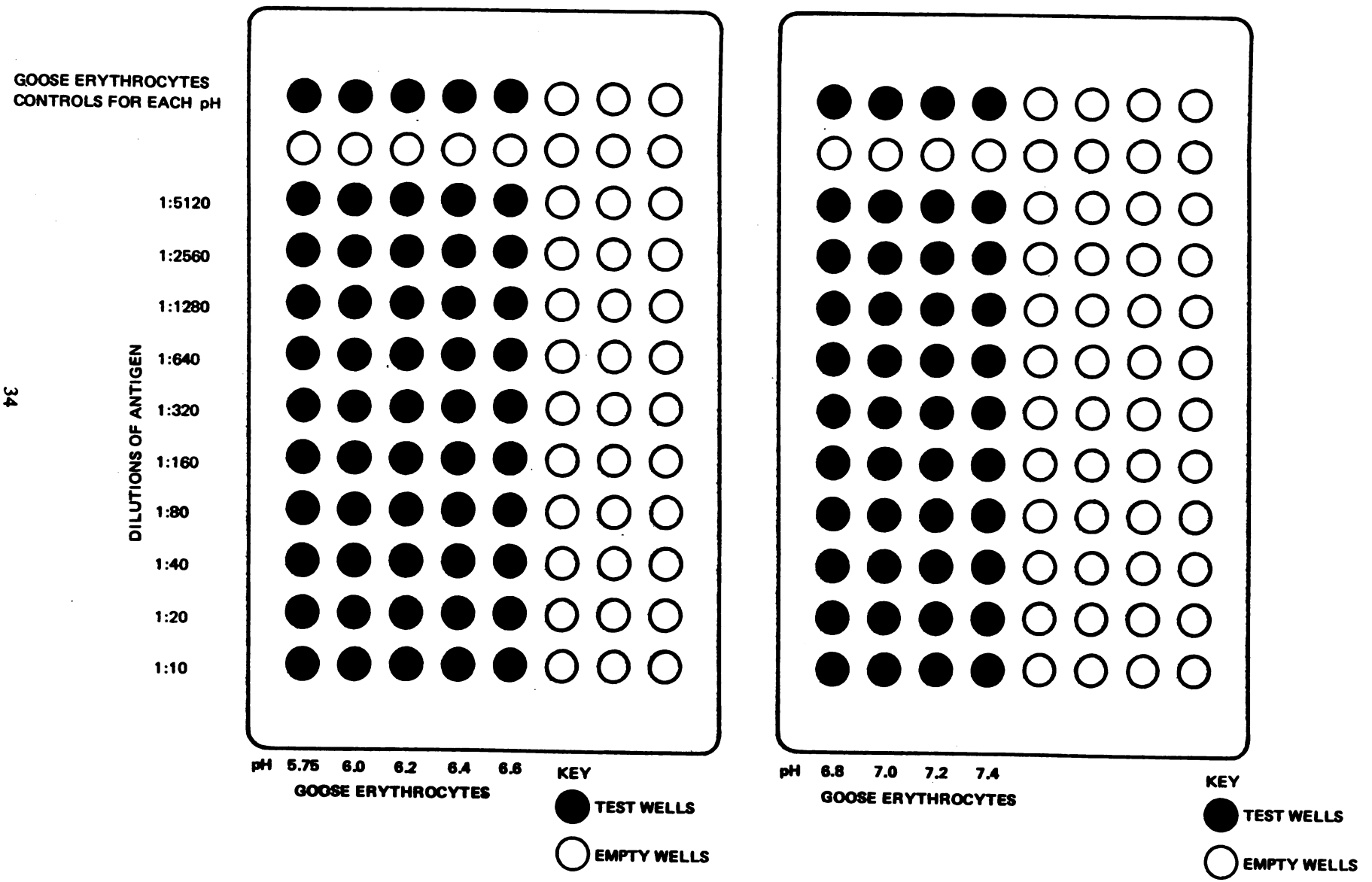
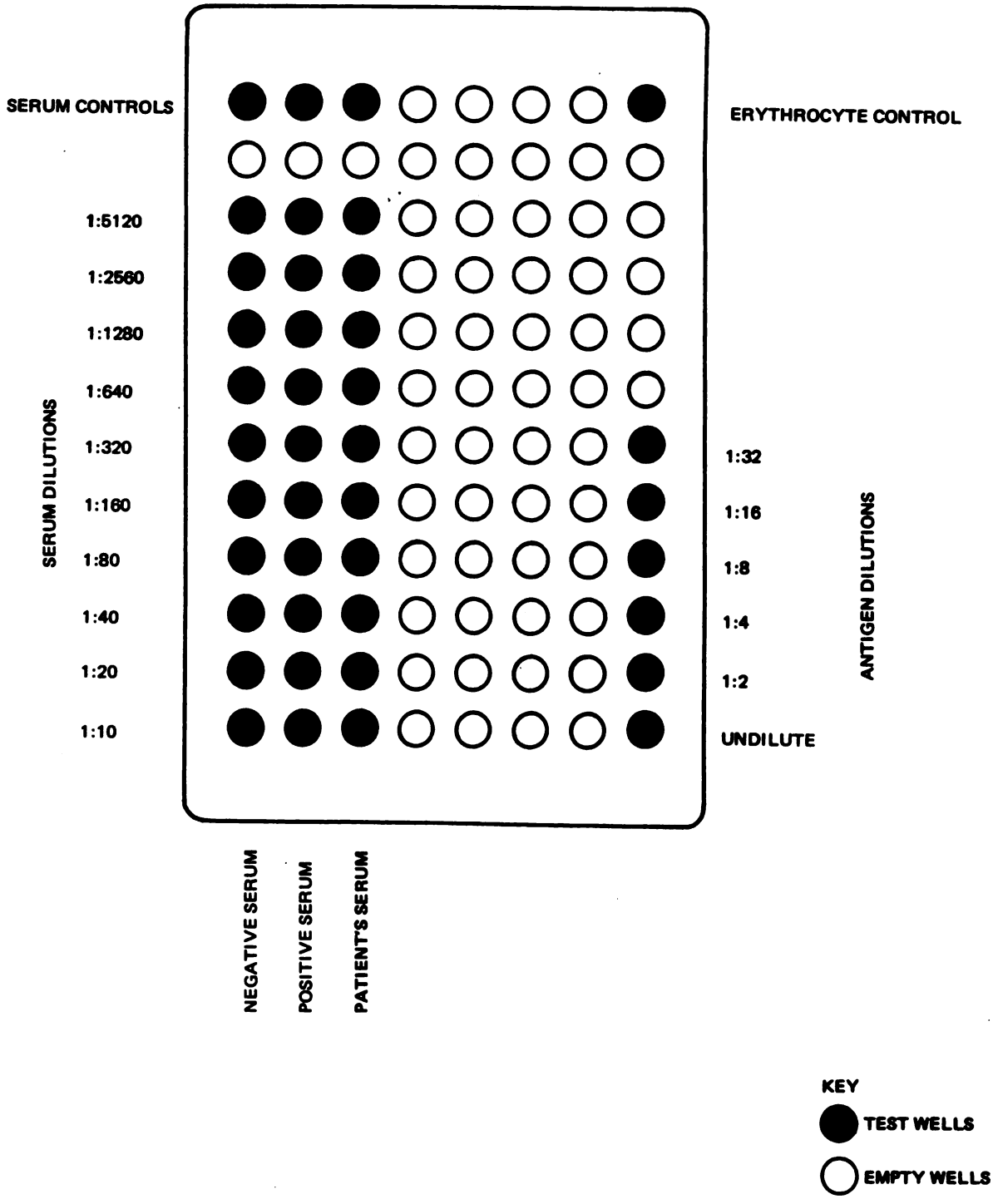


PLATE PATTERN
HEMAGGLUTINATION-INHIBITION TEST
FOR ARBOVIRUSES



i) Add 0.025 ml (eight units) of antigen to each serum-dilution well of the plate.

j) Prepare a **back titration** of each antigen as follows:

(1) Add 0.05 ml (sixteen units) of the test antigen to the first and second of the six **antigen dilution** wells for each antigen that is being titrated.

(2) Add 0.05 ml of BABS with a pipette dropper to wells 2, 3, 4, 5, and 6.

k) Cover the plates with another plate and incubate the tests overnight at 4°C.

l) The next morning, remove the plates. Prepare serial twofold dilutions (1:2 through 1:32) of antigens with 0.05 ml loops. The **antigen dilution** wells now should contain 16, 8, 4, 2, 1, and 0.5 HA units per 0.05 ml.

m) Place the plate on a mechanical shaker and add 0.05 ml of goose erythrocytes which have been suspended in in a 0.2 M phosphate buffer which produces a pH optimal for the hemagglutination of the respective antigens.

n) Seal the plates with cellophane tape and incubate the tests at 37°C for the length of time found optimal for each antigen.

o) Examine the **antigen dilutions** and determine the number of HA units actually used in the tests.

p) Examine the **erythrocyte controls** for the absence of agglutination.

q) Examine the **serum controls** for absence of non-specific agglutination.

r) If all controls are satisfactory, determine the titer of each test serum. The titer of the serum is the highest dilution of serum which completely inhibits agglutination.

4. Precautions and Helpful Suggestions

a. Sera

1) All sera appear to contain nonspecific inhibitors of arbovirus hemagglutination. These are often present to a very high titer and must be removed.

2) Routinely, the kaolin method is used with sera except that from birds. Treatment with kaolin has the great advantage of simplicity and speed but may remove significant amounts of specific anitbody.

3) The kaolin slurry can be held indefinitely at 4°C.

4) Remove the supernatant from the serum - kaolin mixtures very carefully. Fine kaolin particles in the serum may sorb to the antigen, resulting in a false inhibition of hemagglutination.

5) Acetone extraction is currently the method of choice to remove nonspecific inhibitors from avian sera.

6) Acetone extraction may be used on other animal sera if other methods are unsuccessful in removal of nonspecific inhibitors.

7) Extraction with acetone is tedious and not suited to handling of large numbers of specimens.

8) If held at 4°C, acetone extracted sera can be used for many weeks without appreciable changes in titer.

b. **Cells**

1) Red blood cells of adult white geese are now generally accepted as the cell of choice for arbovirus HA and HI tests.

2) Red blood cells from moulting geese do not show proper hemagglutination patterns.

3) Geese may be bled no more than once a month; thirty ml of blood is the maximum amount to be removed. More frequent bleeding results in the presence of immature and fragile cells.

4) Red cells from male geese give better reaction patterns.

5) Goose red blood cells are equally stable in Alsever's and in ACD.

c. **Antigen**

1) Store the 1:10 stock dilution of antigen at 4°C; if sterile, it will last for several years.

d. **Diagnostic test**

1) HI

a) If you are performing tests on a great many sera, screen with the first 2-3 dilutions.

b) When reading results watch for slippage patterns; new plate's may be coated with silicone. The phenomenon is especially bad at high serum concentrations.

c) Use a cell control on each HI plate.

2) Identification of isolates

a) The HI test is the most practical procedure for grouping most arbovirus isolates because of its broad range of activity.

b) Inhibition by either broad Group A or broad Group B serum suggests that the virus is antigenically related to that group.

c) Further characterization of the isolate should be made by the complement fixation and neutralization test against speciric immune sera.

e. A syntron paper jogger may be used as a mechanical vibrator.

5. **Interpretation**

a. In the presence of specific immune serum, arbovirus hemagglutination is inhibited.

b. The titer of the serum is the highest dilution of serum which completely inhibits agglutination.

c. A fourfold difference in titer between an acute and convalescent serum is considered significant and is diagnostic of infection with a virus antigenically related to that used in the test, provided both sera have been prepared and treated in the same manner and tested simultaneously.

d. A single serum specimen may be diagnostic if the titer is very high.

e. HI titers following infection with St. Louis, Western equine, and California group encephalitis viruses are usually higher in the acute specimens than in the convalescent; a fourfold drop in the convalescent specimen is diagnostic.

f. Titers following infection with the virus of Eastern equine encephalitis are higher in the convalescent specimen than in the acute; HI antibodies rise later in the disease.

g. Prior association with yellow fever virus (vaccination or infection) will give a titer of 1:10 to 1:20, occasionally 1:40, with St. Louis encephalitis virus.

h. Serologically positive results with avian sera are suggestive of infection at some undetermined time with an agent antigenically related to the one used in the test.

APPENDIX

Additional Methods for Removal of Nonspecific Inhibitors of Arbovirus Hemagglutination

1. **Treatment of sera: Acetone Extraction**

All sera appear to contain nonspecific inhibitors of arbovirus hemagglutination. These are often present to a very high titer and must be removed. Extraction with acetone is tedious and not suited to handling of large numbers of specimens.

However, if other methods are unsuccessful in removal of nonspecific inhibitors, the acetone procedure will probably yield sera suitable for testing.

a. **Reagents**

1) Avian diagnostic serum
2) Acetone, chilled to 4°C
3) Borate saline, pH 9.0
4) NaCl, 0.9% in distilled water
5) Washed and packed goose erythrocytes

b. **Procedures**

1) Mix 0.2 ml of the avian serum with 1.8 ml of the 0.9% NaCl solution. Use crushed ice bath to chill serum.
2) Add 24 ml of chilled acetone.
3) Mix the solution gently and centrifuge in the cold at 2,000 rpm for 5 minutes. Discard the supernatant.
4) Add 24 ml of acetone to the precipitate and shake vigorously.
5) Centrifuge the serum at 2,000 rpm for 5 minutes. Discard the supernatant.
6) Dry the precipitate under a vacuum.
7) Dissolve the powder in 2 ml of borate saline. Solution is easily affected by leaving the preparation overnight in the refrigerator or for an hour at room temperature with occasional agitation of the tube. This represents a 1:10 serum dilution.
8) Absorb the 1:10 serum dilution with 0.1 ml of washed and packed goose erythrocytes.
9) Place the serum in an ice bath for 20 minutes. Shake the mixture at 5 minute intervals.
10) Centrifuge the serum in the cold at 2500 rpm for 30 minutes and remove the supernatant. The supernatant is ready for use in the HI test.

2. **Treatment of Sera: Protamine Sulfate Extraction**

A nonspecific, acetone-insoluble, arbovirus hemagglutinin inhibitor is present in the sera of adult female chickens and occurs sporadically in serum from birds other than chickens. This procedure for extracting sera with protamine sulfate has been found effective in removing the effects of the inhibitor without substantially decreasing the specific antibodies except in rare cases.

a. **Reagents**

1) Avian diagnostic serum
2) Acetone, chilled to 4°C
3) Protamine sulfate solution, 0.33% in 0.9% NaCl
4) Borate saline, pH 9.0
5) Washed and packed goose erythrocytes

b. Procedures

1) Mix 0.1 ml of the avian serum with 0.3 ml of the 0.33% protamine sulfate solution.
2) Add 12 ml of chilled acetone.
3) Mix the solution gently and centrifuge in the cold at 2,000 rpm for 5 minutes. Discard the supernatant.
4) Add a 12 ml volume of acetone to the precipitate and shake vigorously.
5) Centrifuge the serum at 2,000 rpm for 5 minutes. Discard the supernatant.
6) Dry the precipitate under a vacuum.
7) Dissolve the powder in 1 ml of borate saline. Solution is easily affected by leaving the preparation overnight in the refrigerator or for an hour at room temperature with occasional agitation of the tube. This represents a 1:10 serum dilution.
8) Absorb the 1:10 serum dilution with 0.1 ml of washed and packed goose erythrocytes.
9) Place the serum in an ice bath for 20 minutes. Shake the mixture at 5 minute intervals.
10) Centrifuge the serum in the cold at 2,500 rpm for 30 minutes, and remove the supernatant. The supernatant is ready for use in the HI test.

SEROLOGY OF INFECTIOUS MONONUCLEOSIS

History

Infectious mononucleosis or glandular fever was first described clinically by Pfeiffer in 1889. The first case in the United States was reported in 1896 by West. Original descriptions characterized the entity as a febrile illness accompanied by sore throat and generalized enlargement of lymph nodes. Turk in 1907 was the first to associate abnormal blood cells with the disease; he reported the disease as acute lymphatic leukemia. In 1920 Sprunt and Evans introduced the name infectious mononucleosis and described the unusual cells encountered. In 1923 Downey described the morphological changes in these cells in minute detail. Although Forssman antibodies were described at the turn of the century, it was Paul and Bunnell (1932) who noted that the sera from patients with the clinical and hematological findings of infectious mononucleosis produced clumping (agglutination) of sheep red blood cells. The titer of the sera from infectious mononucleosis patients in most cases is much higher than Forssman antibody found in normal blood sera.

Absorptions of heterophile antibody with guinea pig kidney was first introduced by Forssman (1911) in his basic work on naturally occurring hemolysins and agglutinins of the heterophile or non-specific type. Davidsohn (1930) showed that normal control subjects in serum sickness studies possessed antisheep agglutinins of a heterophile nature. Following this, Baily and Raffel (1935) reported that beef erythrocytes would absorb the sheep agglutinins from the sera of patients with infectious mononucleosis. Davidsohn (1935) introduced the use of differential absorption of sera with boiled guinea pig kidney and boiled beef cell antigens as a diagnostic test for infectious mononucleosis. He demonstrated that the agglutinin present in the sera of patients with infectious mononucleosis remains after absorption by guinea pig kidney antigen but is absorbed by beef cell antigen. The observation by Forssman that blood from cows contained no Forssman type antigen was the basis for the development of the Ox Cell Hemolysin Test as a diagnostic procedure in infectious mononucleosis. This test generally eliminates the false positive reactions.

These serologic tests for infectious monomucleosis are nonspecific; they determine the presence of antibodies that are not formed specifically against the etiologic agent of infectious mononucleosis.

Although the exact etiology of infectious mononucleosis has not been unequivocally established, Henle, et al, in 1968 reported the relation of the Epstein-Barr (EB) virus to this disease. These and other investigators found that antibodies to EB virus developed in patients having had infectious mononucleosis. These antibodies were detectable by an indirect immunoflourescent test. In this test, antibodies are determined with the use of an EB line of Burkitt tumor cells grown on glass cover slips. The FA test for human antibodies to EB virus has been found to be sensitive, reproducible and specific. However, the EB virus antibody is distinctive from the heterophile antibody in that it cannot be absorbed out by sheep or beef red cells and it persists for a longer period of time than do the heterophile antibodies. Due to the persistent FA activity of the EB virus FA test, it appears that this test would be of limited value as a routine diagnostic procedure (Feorino, et al, 1971).

Most authorities agree that the three essential criteria for confirming a diagnosis of infectious mononucleosis are as follows:

1. A clinical picture characterized by sore throat, exudative pharyngotonsillitis, fever, generalized malaise, and lymph node enlargement. Splenomegaly is present in a majority of the cases and hepatitis may occur, but there is rarely marked clinical jaundice.
2. That heterophile antibodies in the serum of patients with infectious mononucleosis can be detected and/or measured serologically.
3. A characteristic blood picture.

Bender (1958) and Hoagland (1960) emphasized the importance of adhering to these criteria, and how the diversion from these criteria has created considerable confusion in the literature.

THE HETEROPHILE ANTIBODY TEST

Forssman (1911) first demonstrated heterophile antibodies when an antibody produced in rabbits against sheep erythrocytes was shown to be toxic to guinea pigs and that the guinea pig kidney possessed antigen which reacted with the rabbit antibody. The term Forssman is limited to these specific heterophile antibodies and antigens. These antigens are present in horses, camels, sheep, mice, dogs, cats, human erythrocytes (Groups A and B), chickens, certain strains of Gram negative bacilli, pneumococcus, parasitic organisms, and fungal organisms. They are absent from the organs and erythrocytes of the rabbit, cow, pig, and rat. The Forssman type is one of many heterophile systems. The terms Forssman antigen and Heterophile antigen have been used synonymously, but the designation "Forssman antigen" should be limited to the antigen discovered by Forssman in guinea pig type tissues. Heterophile antigen should be used to denote a broad group of antigens present in various plants and animals and possessing characteristics similar to those of the Forssman antigen.

In 1932, Paul and Bunnell discovered that sera from patients with clinical and hematological findings of infectious mononucleosis produced clumping (agglutination) of sheep red blood cells. It has been reported that during the first week of illness, only 38 percent of the cases have diagnostically positive titers, but that this increases to 60 percent during the second week, and reaches a peak of 79 percent positive in the third week of the disease. A precipitous decline in heterophile antibody titer then follows in the fourth and subsequent weeks. **The titer bears no relationship to the severity of the disease.**

Davidsohn in 1935-37 and Davidsohn, Stern, and Kashiwagi in 1951 designed a differential test to distinguish between heterophile sheep cell agglutinins in human serum due to: (1) heterophile antigens other than infectious mononucleosis (Forssman "native"); (2) serum sickness; and (3) infectious mononucleosis. This differential test was based on the fact that guinea pig kidney will absorb heterophile sheep cell agglutinins produced by heterophile antigens other than infectious mononucleosis. Beef erythrocytes will absorb the heterophile sheep cell agglutinins produced by infectious mononucleosis or horse serum injection, but not those produced by other heterophile antigens.

Serum to be tested for heterophile sheep cell agglutinins is set up in dilutions usually beginning at 1:5 and continuing in a two-fold manner. Sheep erythrocytes are added, and following incubation the test is read for agglutination. This is known as the Presumptive Test, and only determines whether or not agglutinins to sheep erythrocytes are present. If, in this test, heterophile sheep cell agglutinins are present in a titer of 1:40 or higher, it is then necessary to differentiate the agglutinins of infectious mononucleosis from the other non-specific reactions.

The differential absorption test of Davidsohn consists of absorbing serum with both a 2.0% suspension of boiled guinea pig or horse kidney antigen and a 2.0% suspension of boiled beef cell antigen. The differential test for infectious mononucleosis is positive if the titer of heterophile sheep cell agglutinins after guinea pig or horse kidney adsorption is not more than three tubes or doubling dilutions below the presumptive titer, and if the titer after beef cell absorption is negative or a least three twofold dilutions lower than the presumptive titer.

Absorption Patterns for Various Heterophile Antibodies*

Type of Heterophile Antibody	Absorbed by Guinea Pig Kidney	Absorbed by Beef Erythrocytes
Forssman ("native")	Yes	No or partial
Serum Sickness	Yes	Yes
Infectious Mononucleosis	No or slight	Yes

* Bennett, C. W., Clinical Serology, Charles C. Thomas, Publisher, Springfield, Illinois, 1964. Page 173.

The test for heterophile antibodies is of confirmatory diagnostic value in cases of infectious mononucleosis with typical clinical and hematological features. It is also of importance to rule out infectious mononucleosis in cases where similar clinical and/or hematological features are encountered with other diseases such as leukemia.

I. Paul-Bunnell Presumptive Test

A. Materials

1. Reagents

a. Veronal buffered diluent (VBD)
b. Sheep erythrocytes
c. Sera
   1) Positive
   2) Negative
   3) Patients'

2. Equipment

a. Serologic tubes for preparing initial serum dilutions.
b. Disposable pipettes (0.5 ml or 1.0 ml)
c. 0.025 ml microduluters
d. 0.025 ml microtitration pipette droppers
e. "V" microtitration plates

B. Methods

1. Preparation of Reagents and Materials

a. Preparation of veronal buffered diluent (VBD)

1) Stock buffer solution (concentrated five times)

a) Combine the following in a 2 liter volumetric flask in the order listed:

Distilled Water	1500 ml
NaCl	83.00 gm
Na-5, 5-diethyl barbituate	10.19 gm
1 N hydrochloric acid	34.58 ml
Stock solution containing 1 molar $MgCl_2$ and 0.3 molar $CaCl_2$ (20.3 gm $MgCl_2 \cdot 6\ H_2O$ and 4.4 gm $CaCl_2 \cdot 2\ H_2O$ in 100 ml distilled water)	5.00 ml

b) Fill to the 2.0 liter mark with distilled water. Mix thoroughly.

c) Check the pH of the stock buffer before refrigeration by making a 1:5 dilution with distilled water. The pH of the diluted stock must be 7.3 to 7.4. If the pH is not in this range, discard and prepare fresh stock buffer.

2) Preparation of gelatin-water solution

a) Add 1.0 gm of gelatin to 100 ml of distilled water. Bring to boil to insure solution of gelatin.

b) Make up to 800 ml with distilled water at room temperature.

c) Chill in refrigerator. (This solution should not be held longer than 1 week to avoid contamination.)

3) Preparation of VBD for daily use (containing 0.1% gelatin)

Add 4 volumes of gelatin water to 1 volume of stock buffer. Store in the refrigerator. VBD should not be stored longer than 24 hours.

NOTE: The pH of the VBD must be 7.3 to 7.4.

b. Preparation of 2% Red Cell Suspension

There are several methods of preparing relatively accurate suspensions of red blood cells. The two most commonly used are the centrifugation and spectrophotometric methods. The centrifugation method measures cell mass by volume, whereas the spectrophotometric measures the amount of released hemoglobin which in turn is related to cell mass. The spectrophotometric method is more accurate and reproducible and is considered the method of choice.

1) Washing cells

   a) Add 2 or 3 volumes of cold VBD to each volume of preserved cells and centrifuge at 600 X G for 5 minutes. This force can be obtained by centrifugation at 2,000 rpm in an SB-1 International Centrifuge with a head having a radius of 13 cm., or at 1,700 rpm in an SB-2 International Centrifuge with a head having a radius of 19 cm.

   b) Carefully remove supernatant fluid and the white cell layer by suction without disturbing the erythrocytes.

   c) Fill centrifuge tube again with cold VBD. Thoroughly resuspend the cells by gently mixing with a pipette. Centrifuge at 600 X G for 5 minutes, and repeat the process for a total of three washings. If the supernatant is not colorless after the second washing, cells are too fragile and must not be used.

   d) Resuspend the cells once more in cold VBD and centrifuge for 10 minutes at 600 X G to pack the cells.

   e) Record the volume of packed cells in the centrifuge tube and remove the supernate. Care should be taken to remove as much fluid as possible without disturbing the cells.

2) Standardization of 2.0 percent cell suspension by centrifugation.

   a) Prepare a 2.0% cell suspension by adding 49 volumes of VBD to 1 volume of packed red cells.

   b) To check the density of the 2.0% cell suspension, pipette 10 ml into a 12 or 15 ml graduated centrifuge tube and centrifuge at 600 X G for 10 minutes. A 10.0 ml aliquot of a properly prepared cell suspension should produce 0.2 ml of packed cells.

      NOTE: The accuracy of the graduated centrifuge tube should be predetermined.

   c) When the volume of packed cells is under or above the 0.2 ml point, the cell suspension should be adjusted. The quantity of VBD which must be added to or removed from the cell suspension is determined by the following formula:

      Corrected volume of cell suspension =

      $$\frac{\text{Actual reading of centrifuge tube}}{\text{Correct reading of centrifuge tube}} \times \text{volume of cell suspension}$$

      NOTE: Keep the cell suspension in the refrigerator when not in use. Always shake the flask gently before use to secure an even suspension of the erythrocytes which settle on standing.

2. **Performance of Test**

   a. Make a 1:5 dilution of each serum to be tested and inactivate at 56℃ for 30 minutes. 0.1 ml of serum plus 0.4 ml VBD will be adequate to provide sufficient material for duplicate examinations.

   b. Mark off "V" plates so that each serum is assigned two rows of 10 wells each. (See pattern - page 49)

   c. Add 0.025 ml of buffer to all wells in each row except the first well. Add 0.025 ml of buffer to a well designated "cell control."

   d. Add 0.05 ml of the 1:5 serum dilution to the first well in each of the two rows for each respective serum.

   e. Use a pretested 0.025 ml microtitration diluter to mix and transfer serum in the series of wells in each row.

   f. Add 0.025 ml of the 2% suspension of sheep red blood cells (SRBC) including the cell control and shake each plate on a mechanical vibrator to mix the contents.

   g. Seal each plate with transparent plastic tape.

   h. Incubate at 37℃ for 2 hours, read for agglutination, and place at 4℃ overnight.

   i. The next morning remove the plates from the refrigerator, incubate at 37℃ for 15 minutes to elute cold agglutinins if present, and read for agglutination to determine the final serum titer.

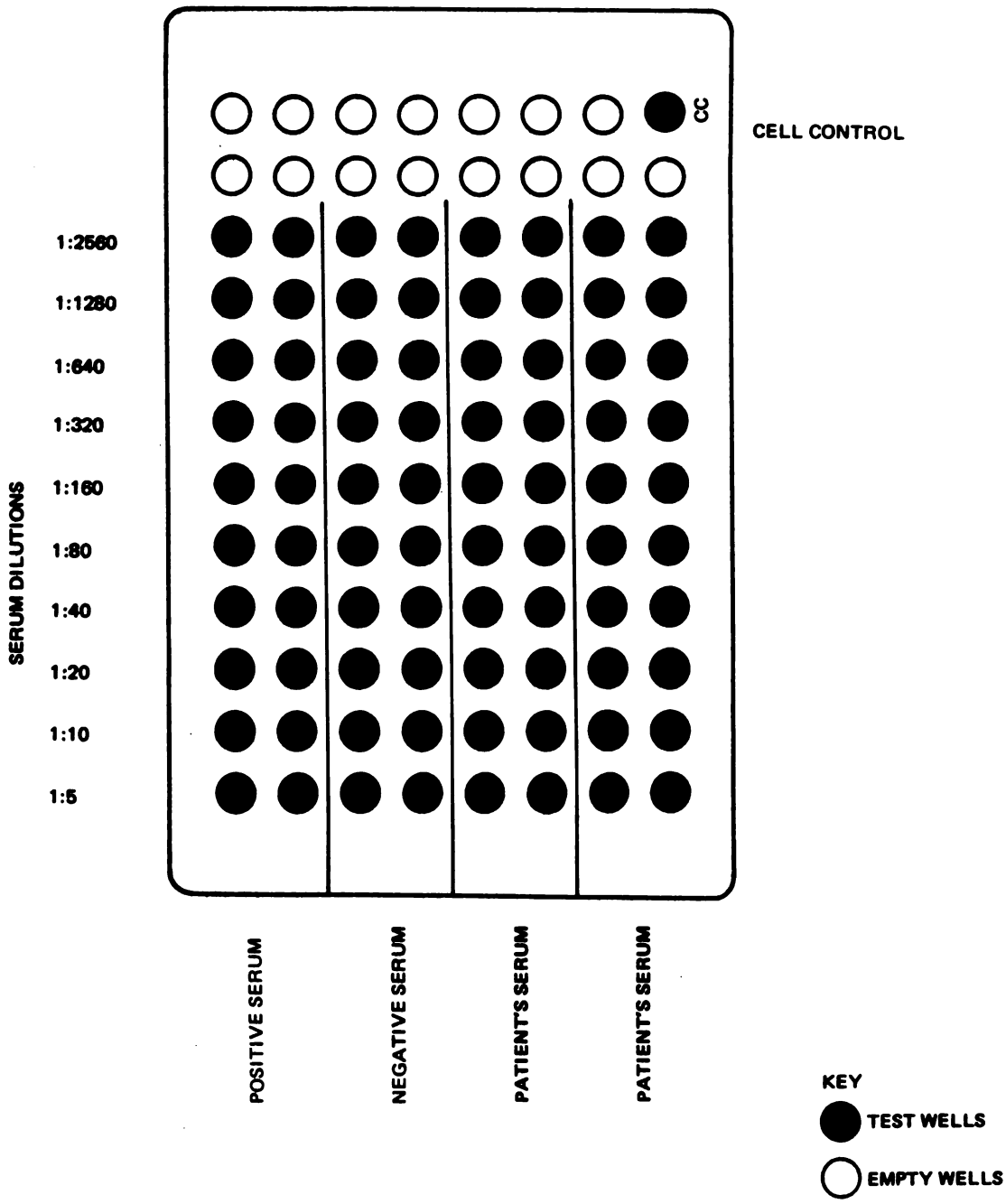
   j. Test may be read by standing plates on end and reading by the "tear drop" or "streaming method." (Agglutinated cells will adhere in one mass, while non-agglutinated cells will run in a fashion similar to a tear drop.)

C. **Interpretation of Results**

Specimens which contain agglutinins in a dilution of 1:40 or higher are considered positive in the Presumptive Test. In order to differentiate the type of heterophile antibody present, the Davidsohn Differential Test must be performed.

*There is not general agreement at this point in the protocol. The variations performed by many laboratories include: (1) incubation at room temperature (instead of 37℃) for 2 hours; (2) incubation for 1 hour at 37℃ or room temperature (RT) and then at 4℃ overnight; and (3) following the overnight incubation at 4℃ the plates are equilibrated by placing them at RT instead of 37℃.

PLATE PATTERN FOR PAUL-BUNNELL PRESUMPTIVE TEST FOR INFECTIOUS MONONUCLEOSIS



II. The Davidsohn Differential Test

A. Materials

1. Reagents

a. Veronal buffered saline (VBD)
b. Sheep erythrocytes
c. Horse Kidney or guinea pig kidney absorption antigen
d. Beef cell absorption antigen
e. Sera
   1) Positive
   2) Negative
   3) Patient's

2. Equipment

a. Serologic tubes for preparing dilutions and performing adsorptions
b. Disposable pipettes (0.5 ml or 1.0 ml)
c. 0.025 ml microdiluters
d. 0.025 microtitration pipette droppers
e. "V" microtitration plates

B. Methods

1. Preparation of Reagents and Materials

a. Preparation of veronal buffered diluent (VBD)

1) Stock buffer solution (concentrated five times)

a) Combine the following in a 2 liter volumetric flask in the order listed:

Distilled Water	1500 ml
NaCl	83.00 gm
Na-5, 5-diethyl barbituate	10.19 gm
1 N hydrochloric acid	34.58 ml
Stock solution containing 1 molar $MgCl_2$ and 0.3 molar $CaCl_2$ (20.3 gm $MgCl_2 \cdot 6\ H_2O$ and 4.4 gm $CaCl_2 \cdot 2\ H_2O$ in 100 ml distilled water)	5.00 ml

b) Fill to the 2.0 liter mark with distilled water. Mix thoroughly.

c) Check the pH of the stock buffer before refrigeration by making a 1:5 dilution with distilled water. The pH of the diluted stock must be 7.3 to 7.4. If the pH is not in this range, discard and prepare fresh stock buffer.

2) Preparation of gelatin-water solution

a) Add 1.0 gm of gelatin to 100 ml of distilled water. Bring to boil to insure solution of gelatin.

b) Make up to 800 ml with distilled water at room temperature.

c) Chill in refrigerator. (This solution should not be held longer than 1 week to avoid contamination.)

3) Preparation of VBD for daily use (containing 0.1% gelatin)

Add 4 volumes of gelatin water to 1 volume of stock buffer. Store in the refrigerator. VBD should not be stored longer than 24 hours.
NOTE: The pH of the VBD must be 7.3 to 7.4.

b. Preparation of 2% Red Cell Suspension

There are several methods of preparing relatively accurate suspensions of red blood cells. The two most commonly used are the centrifugation and spectrophotometric methods. The centrifugation method measures cell mass by volume, whereas the spectrophotometric measures the amount of released hemoglobin which in turn is related to cell mass. The spectrophotometric method is more accurate and reproducible and is considered the method of choice.

1) Washing cells

   a) Add 2 or 3 volumes of cold VBD to each volume of preserved cells and centrifuge at 600 x G for 5 minutes. This force can be obtained by centrifugation at 2,000 rpm in an SB-1 International Centrifuge with a head having a radius of 13 cm., or at 1700 rpm in an SB-2 International Centrifuge with a head having a radius of 19 cm.

   b) Carefully remove supernatant fluid and the white cell layer by suction without disturbing the erythrocytes.

   c) Fill centrifuge tube again with cold VBD. Thoroughly resuspend the cells by gently mixing with a pipette. Centrifuge at 600 x G for 5 minutes, and repeat the process for a total of three washings. If the supernatant is not colorless after the second washing, cells are too fragile and must not be used.

   d) Resuspend the cells once more in cold VBD and centrifuge for 10 minutes at 600 x G to pack the cells.

   e) Record the volume of packed cells in the centrifuge tube and remove the supernate. Care should be taken to remove as much fluid as possible without disturbing the cells.

2) Standardization of 2.0 percent cell suspension by centrifugation

   a) Prepare a 2.0% cell suspension by adding 49 volumes of VBD to 1 volume of packed red cells.

   b) To check the denisty of the 2.0% cell suspension, pipette 10 ml into a 12 or 15 ml graduated centrifuge tube and centrifuge at 600 x G for 10 minutes. A 10.0 ml aliquot of a properly prepared cell suspension should produce 0.2 ml of packed cells.

      NOTE: The accuracy of the graduated centrifuge tube should be predetermined.

   c) When the volume of packed cells is under or above the 0.2 ml point, the cell suspension should be adjusted. The quantity of VBD which must be added to or removed from the cell suspension is determined by the following formula:

      Corrected volume of cell suspension =

$$\frac{\text{Actual reading of centrifuge tube}}{\text{Correct reading of centrifuge tube}} \times \text{Volume of cell suspension}$$

      NOTE: Keep the cell suspension in the refrigerator when not in use. Always shake the flask gently before use to secure an even suspension of the erythrocytes which settle on standing.

2. **Performance of Test**

a. Reconstitute the absorbing antigens to the volume recommended by the manufacturer.

b. For serum absorption, place 0.8 ml of each absorbing antigen in tubes and add 0.2 ml of inactivated serum. Mix well by agitation.

c. Allow the mixture to stand for 3 minutes. Centrifuge at 600 G for 10 minutes.

d. Carefully transfer the supernatant fluid of each tube, with a capillary pipette, to another labelled tube. The supernate fluid is considered a 1:5 serum dilution.

e. Mark off two "V" plates so that each serum is assigned two rows of 10 wells each (See pattern - page 53). One plate will be for horse kidney absorption and the other for beef cell absorption. Be sure each plate is properly identified.

f. Add 0.025 ml of buffer to all cups in each row except the first well. Add 0.025 ml of buffer to a cup designated "cell control."

g. Add 0.05 ml of the 1:5 absorbed serum dilution to the first well in each of the two rows for each respective serum.

h. Use a pretested 0.025 ml microtitration diluter to mix and transfer serum in the series of wells in each row.

i. Add 0.025 ml of the 2% suspension of SRBC to each well including the cell control. Shake each plate on a mechanical vibrator to mix the contents.

j. Seal each plate with transparent plastic tape.

k. Incubate at 37°C for 2 hours, read for agglutination, and place at 4°C overnight.

l. The next morning remove the plates from the refrigerator, incubate at 37°C for 15 minutes to elute cold agglutinins, if present, and read for agglutination to determine final serum endpoint.

C. **Interpretation of Results**

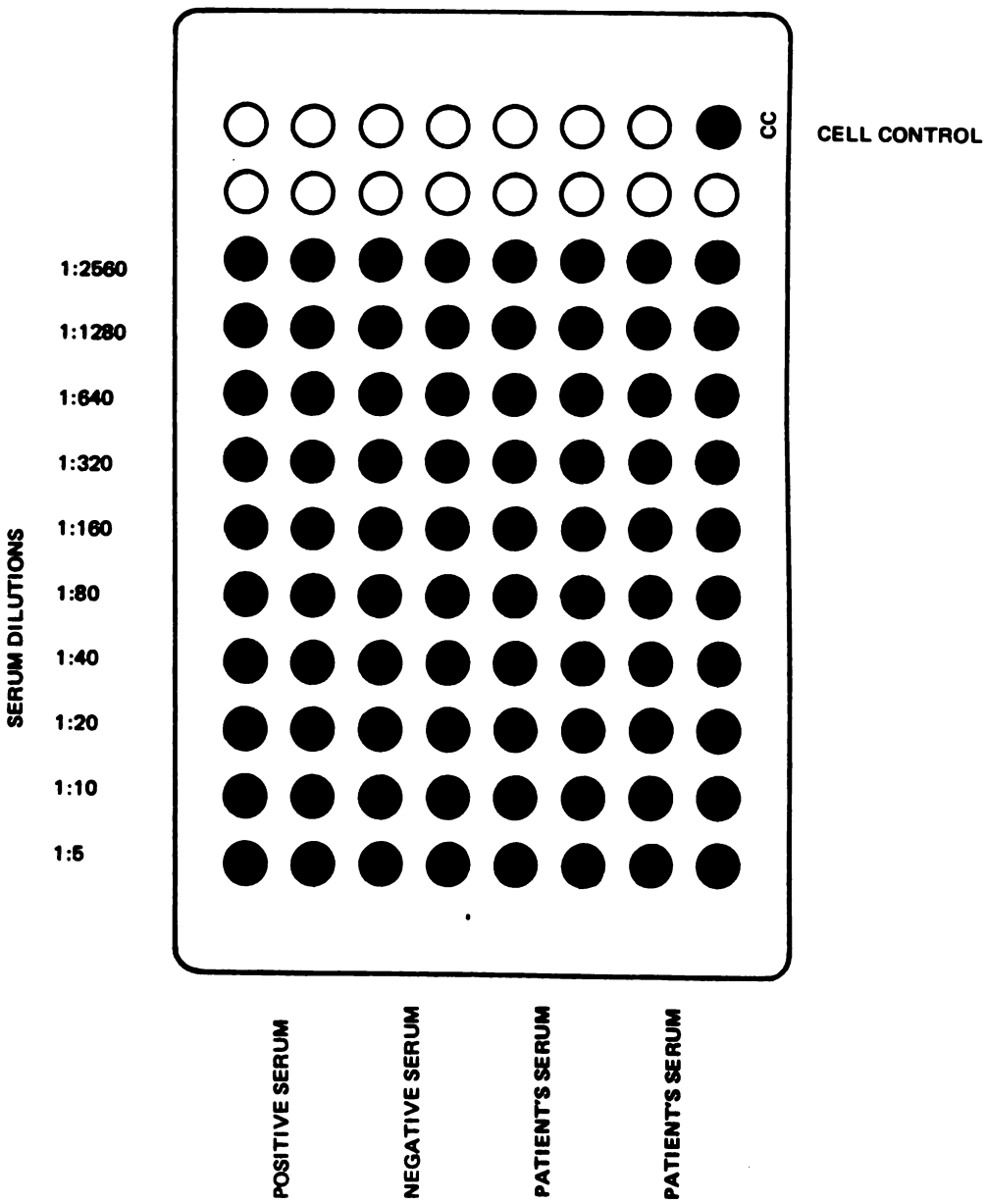
The following table summarizes the absorption patterns for heterophile antibodies of the Forssman, serum sickness, infectious mononucleosis, and other (undescribed) types.

ABSORPTION PATTERNS FOR VARIOUS HETEROPHILE ANTIBODIES

Type of Heterophile Antibody	Absorbed by Guinea Pig or Horse Kidney	Absorbed by Beef RBC's
Forssman	Yes	No or partial
Serum sickness	Yes	Yes
Infectious mononucleosis	No or slight*	Yes*
Other (undescribed)	No or slight	No or partial

*The Differential Test for infectious mononucleosis is positive if the titer of heterophile sheep cell agglutinins after absorption with guinea pig kidney or horse kidney is not more than three dilutions lower, and at the same time the titer of heterophile sheep cell agglutinins after absorption with beef erythrocytes is at least three dilutions lower than the titer in the Presumptive Test.

PLATE PATTERN FOR DAVIDSOHN DIFFERENTIAL TEST FOR INFECTIOUS MONONUCLEOSIS



The Ox Cell Hemolysin Test

Bailey and Raffel (1935) reported the occurrence of high titered ox cell hemolysin in the sera of patients with infectious mononucleosis while normal sera from adults had a very low incidence of hemolysins to ox cells. Mason (1951) applied the ox cell hemolysin test to the diagnosis of infectious mononucleosis and reported that it was more specific than the classical heterophile antibody (Paul and Bunnel, 1932, Beer, 1936) test with absorptions (Davidsohn, 1937). Mason and Leyton (1952) also observed that the ox cell hemolysin appeared earlier than the sheep cell agglutinin and remained elevated longer.

Technically, the ox cell hemolysin test has several advantages over the Davidsohn Differential absorption technique. A titer of 1:40 is clinically significant in 95% of sera tested. The test is incubated and read in thirty minutes and is, therefore, less time consuming than the Davidsohn Differential Test. If the titer of a serum specimen collected early in the course of clinical illness is 1:20 or less, another serum specimen should be tested in 7 to 10 days. The ox cell hemolysin test has been reported to give false reactions rarely, while in both the Presumptive and Differential tests, false negative and false positive results may occur. As in all serological diagnostic tests, demonstration of a rise in titer adds confidence to serological confirmation of clinical illness.

In the past, other workers (Eyquem, 1961; Eyquem, 1959; Mikkelsen, 1958; and Peterson, 1956) have proposed the clinical use of the ox cell hemolysin test. Several technical difficulties were noted in these previous reports.

> Titers reported have been on the basis of final dilutions rather than the serum dilution.
>
> The amount of complement used has varied.
>
> The use of fresh complement has produced false positive titers in normal sera.
>
> The sera has been diluted with saline containing magnesium sulfate without buffer to control the pH.

The ox cell hemolysin test presented here avoids some of these difficulties and has been found to be suitable for clinical use.

A. Materials

1. Reagents

a. Veronal buffered diluent (VBD)
b. Veronal buffered water
c. 2% ox erythrocyte suspension
d. Sera
    1) Positive
    2) Negative
    3) Patients'
e. Complement, lyophilized

2. **Equipment**

a. "U" microtitration plates
b. 0.025 ml microtitration diluters
c. 0.025 ml microtitration pipette droppers
d. Disposable pipettes (0.5 ml or 1.0 ml)
e. Serologic tubes for preparing initial serum dilutions
f. 37℃ water bath
g. 56℃ water bath
h. Mechanical vibrator
i. Centrifuge that will accomodate microtiter centrifuge carriers (Cooke Engineering Co.)
j. Beaker for ice to keep complement chilled
k. pH meter

B. Methods

1. Preparation of Reagents and Materials

a. Preparation of veronal buffered diluent (VBD)

1) Stock buffer solution (concentrated five times)

a) Combine the following in a 2 liter volumetric flask in the order listed:

Distilled Water	1500 ml
NaCl	83.00 gm
Na-5, 5-diethyl barbituate	10.19 gm
1 N hydrochloric acid	34.58 ml
Stock solution containing 1 molar $MgCl_2$ and 0.3 molar $CaCl_2$ (20.3 gm $MgCl_2 \cdot 6\,H_2O$ and 4.4 gm $CaCl_2 \cdot 2\,H_2O$ in 100 ml distilled water)	5.00 ml

b) Fill to the 2.0 liter mark with distilled water. Mix thoroughly.

c) Check the pH of the stock buffer before refrigeration by making a 1:5 dilution with distilled water. The pH of the diluted stock must be 7.3 to 7.4. If the pH is not in this range, discard and prepare fresh stock buffer.

2) Preparation of gelatin-water solution.

a) Add 1.0 gm of gelatin to 100 ml of distilled water. Bring to boil to insure solution of gelatin.

b) Make up to 800 ml with distilled water at room temperature.

c) Chill in refrigerator. (This solution should not be held longer than 1 week to avoid contamination.)

3) Preparation of VBD for daily use (containing 0.1% gelatin)

Add 4 volumes of gelatin water to 1 volume of stock buffer. Store in the refrigerator. VBD should not be stored longer than 24 hours.
NOTE: The pH of the VBD must be 7.3 to 7.4.

b. Preparation of Veronal Buffered Water

1) Stock buffer solution

a) Combine reagents in a 1 liter volumetric flask in the order listed:

Distilled water	300-400 ml
Na-5, 5-diethyl barbituate	5.1 gm
1 N hydrochloric acid	17.25 ml

b) Fill to the 1.0-liter mark with distilled water.

c. Preparation of 2% ox cell suspension

1) Prepare a 2.0% cell suspension by adding 49 volumes of VBD to 1 volume of packed red cells.

2) To check the density of the 2.0% cell suspension, pipette 10 ml into a 12 or 15 ml graduated centrifuge tube and centrifuge at 600 X G for 10 minutes. A 10.0 ml aliquot of a properly prepared cell suspension should produce 0.2 ml of packed cells.

NOTE: The accuracy of the graduated centrifuge tube should be predetermined.

3) When the volume of packed cells is under or above the 0.2 ml point, the cell suspension should be adjusted. The quantity of VBD which must be added to or removed from the cell suspension is determined by the following formula:

Corrected volume of cell suspension =

$$\frac{\text{Actual reading of centrifuge tube}}{\text{Correct reading of centrifuge tube}} \times \text{volume of cell suspension}$$

NOTE: Keep the cell suspension in the refrigerator when not in use. Always shake the flask gently before use to secure an even suspension of the erythrocytes which settle on standing.

d. Reconstitution of lyophilized (lyo) commercial complement

1) Allow the diluent and the lyo-complement vials to come to room temperature.

2) Remove the crimps from the vials very carefully and slowly to insure that the lyo complement does not escape from the vials.

3) Pipette the diluent slowly into the complement. Do not splash. To reconsitutue 7 ml vials, pipette 5 ml of diluent. To reconstitute 20.00 ml vials, pipette 15 ml of diluent.

4) Allow the complement to stand at 4℃ until all large clumps have dissolved. This will take 1-2 hours.

5) Place the complement in a 4℃ refrigerator overnight.

6) Prepare a 1:15 dilution (using cold VBD) for use in the ox cell hemolysin test.

2. **Performance of Test**

a. Make a 1:10 dilution of each serum (0.1 ml undiluted serum + 0.9 ml VBD) and inactivate at 56℃ for 30 minutes.

b. Mark off a "U" plate so that each serum is assigned two rows of 10 wells each. (See pattern, page page 57.)

c. Add 0.025 ml of buffer (VBD) to wells two through ten in each row, 0.05 ml of VBD to a well designated "cell control," and 0.025 ml of VBD to a well designated "complement-ox cell control."

d. Add 0.05 ml of the 1:10 serum dilution to the first well in each of the two rows for each respective serum.

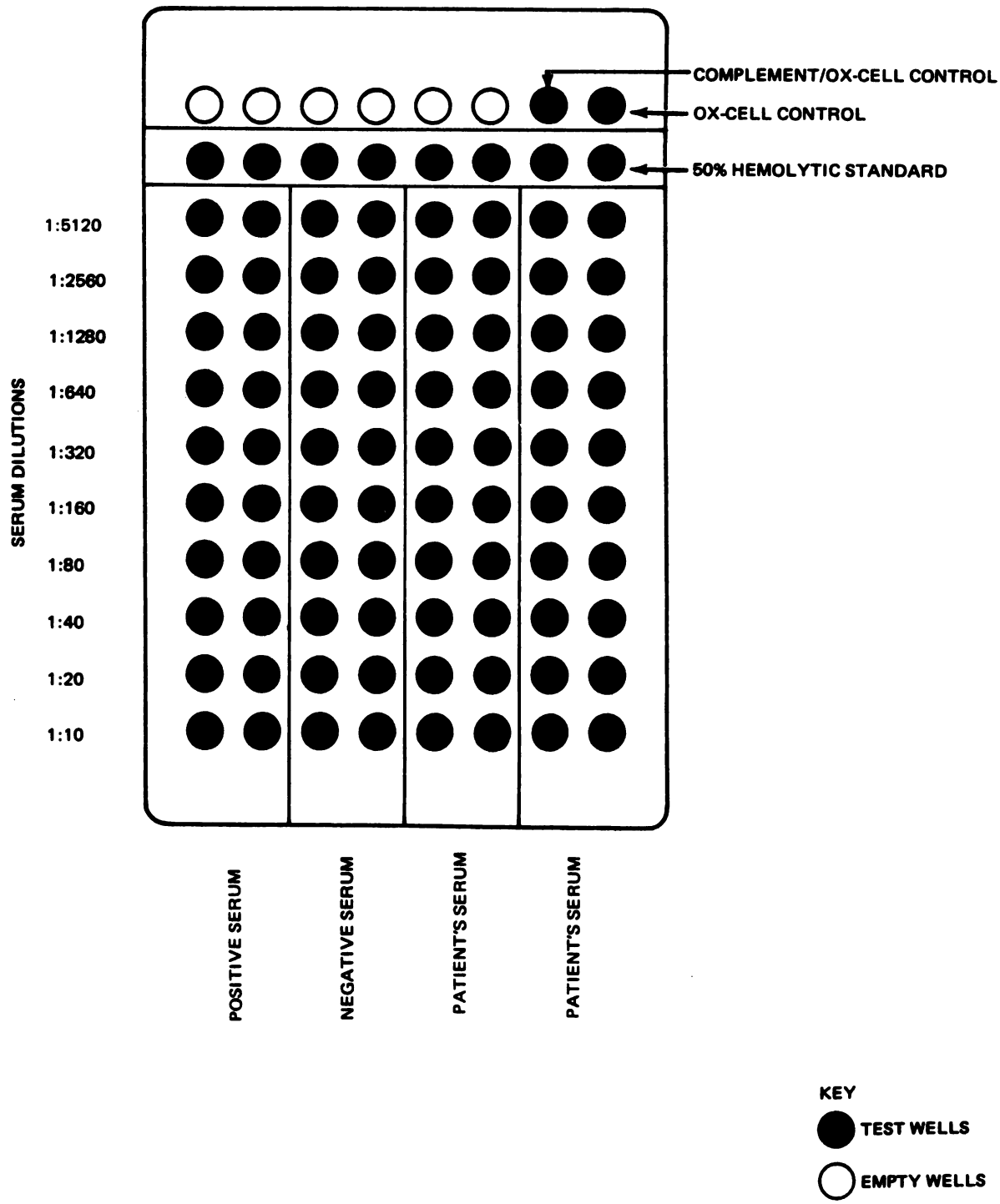
e. Use a pretested 0.025 ml microtitration diluter to mix and transfer serum in the series of wells in each row.

f. Prepare a 50% hemolytic end point standard by adding the following reagents, in the order listed, to a 12 x 75 mm test tube.

1) 0.25 ml of a 2% ox cell suspension
2) 1.0 ml of buffered water
3) 0.25 ml of diluted (1:15) complement

Transfer 0.075 ml (with a pipette or three drops with a 0.025 ml micro-dropper) to each well in column 11 of the "U" plate.

PLATE PATTERN FOR THE OX CELL HEMOLYSIN TEST



g. With a 0.025 ml micro-dropper, add 0.025 ml of the 2% ox cell suspension to each well **except** the 50% hemolytic standards in column 11.

h. Similarly, add 0.025 ml of a 1:15 dilution of complement to each well **except** the 50% hemolytic standards in column 11 and the well marked "cell control."

i. Shake each plate on a mechanical vibrator to mix the contents.

j. Seal each plate with a transparent plastic tape.

k. Incubate plate at 37°C for 30 minutes by floating it in a 37°C water bath.

l. Centrifuge at 150 G for 2 minutes.

m. Read and record results.

C. Interpretation of Results

The serum titer end point is the well that most nearly matches the color of the 50% end point standard in column 11. In the absence of horse serum injection, a titer of 1:40 or greater is serologic evidence in 95% of the patients with infectious mononucleosis.

The ox cell control and the complement-ox cell control tubes should not be hemolyzed (0% hemolysis).

COMMERCIAL RAPID SLIDE TESTS

There are several commercial kits available for the detection of heterophile antibodies in infectious mononucleosis. They do not all operate on the same principle and for that reason a review of the published literature leading to the development of these slide tests would be beneficial.

The basic research by Forssman (1911), Davidsohn (1930), Paul and Bunnell (1932), and Bailey and Raffel (1935) has been discussed previously. In their investigations, Stuart, Griffin, Wheeler, and Battey (1936) and Barrett (1941) demonstrated that horse erythrocyte antigen also reacted with serum from infectious mononucleosis cases.

Since other heterophile systems exist as demonstrated by Davidsohn (1930), the Paul-Bunnell presumptive test was not adequate for distinguishing the Forssman, serum sickness, infectious mononucleosis, and other heterophile agglutinins. Davidsohn (1937 and 1938) reported differential methods for the separation of serum sickness, infectious mononucleosis and other heterophile antibodies and Davidsohn, Stern, and Kashiwagi (1951) introduced the present differential test for infectious mononucleosis.

The differential test aided in the detection and separation of infectious mononucleosis heterophile antibodies from others, but a need for a more direct and simpler test remained. Wollner (1955) found that papain, a proteolytic enzyme, would destroy the receptor sites on the erythrocyte and in the same year (1955) published a test for infectious mononucleosis using papain treated and native red cells. This work was confirmed and broadened to include other receptor destroying substances by Springer and Rapaport (1957) and confirmed again by Muschel and Piper (1959).

Cox and Vermillion (1956), using preserved, formalin treated sheep red blood cells, developed a rapid slide test for infectious mononucleosis, but the use of non-treated sheep cells in presumptive rapid slide tests dates back to Butt and Foord (1935), Straus (1936), Rappaport and Skaritov (1949), Maloney and Malzone (1949), Vaughn (1951), and Brumfitt and O'Grady (1957). Lovric (1961) reported a slide test using papain treated cells as reported Wollner (1955). By using both native and papain treated erythrocytes (sheep or horse) he reported that this obviated the need for specific absorption antigens as was described in the Davidsohn Differential Test. Another variation in the rapid slide test was the use of stabilized, formalin treated horse red blood cells in a presumptive rapid slide test as reported and evaluated by Hoff and Bauer (1965). This report claims a low incidence of false positives using this test.

In summarizing the research efforts of many investigations in the development of serologic tests for infectious mononucleosis, several important principles have evolved on which rapid slide tests have been based. These are:

1. That patients with infectious mononucleosis develop sheep erythrocyte agglutinins.

2. That patients with infectious mononucleosis develop horse erythrocyte agglutinins.

3. Sheep or horse erythrocytes that are treated with formalin become much more stable and can be used instead of non-treated cells with no loss of sensitivity.

4. The use of tissue suspensions and extracts (guinea pig or horse kidney and beef erythrocytes) to absorb the heterophile agglutinins in an effort to differentiate infectious mononucleosis from other heterophile systems. There are slide test versions of the Davidsohn Differential Tube Test.

5. The use of proteolytic enzymes, i.e., papain, for removing the infectious mononucleosis receptor sites from the erythrocytes.

Since the protocols for the various kits are different, the directions for each must be followed and the interpretation made according to each commercial company's printed brochures.

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Singer-Plotz Latex Fixation Test

Rheumatoid arthritis is a systemic disease of the connective tissues, resulting from an immune response by the host to one or more specific determinants present in his own gamma globulin. The most frequent site of injury in adults is the synovial lining of joints. This immune response frequently can be detected by demonstrating serologically the presence of "rheumatoid factors" (abbreviated RF or RA) in a patient's serum. In addition to rheumatoid arthritis, these factors have been found in the serum of patients with periarteritis nodosa, osteoarthritis, fibrositis, and spondylitis rheumatica.

Rheumatoid factors are proteins of high molecular weight composed of complex gamma globulin with sedimentation coefficents of 19 S and 7 S. These factors begin to occur in the serum several months after the beginning of the rheumatoid arthritis process and may persist at a practically constant level for months or years regardless of therapy. The technique most commonly used today for the detection of rheumatoid factors is based on a latex tube agglutination test first described by Singer and Plotz in 1956.

The method of Singer and Plotz involves the use of immune globulin coated latex particles for detecting rheumatoid factor.

1. **Principles**

   The Singer-Plotz Latex Fixation Test for "rheumatoid factor" is based on the following principles:

   a. Gamma globulin can be adsorbed onto latex particles, providing an optical indicator of the serological reaction.

   b. "Rheumatoid factor" in the patient's serum reacts with gamma globulin on the surface of latex particles, resulting in visible agglutination.

   c. The quantity of "rheumatoid factor" in the patient's serum can be determined by adding a constant volume of latex-gamma globulin mixture to serial dilutions of the serum. The antibody titer is an expression of the serum dilution containing just enough "rheumatoid factor" to agglutinate the globulin coated particles.

2. **Materials**

   a. **Reagents**

      1) Borate saline buffer, pH 8.2
      2) Polystyrene latex suspension (1% stock suspension)
      3) Gamma globulin solution (0.5% stock solution)
      4) Antisera:
         Positive serum of known titers
         Normal serum for negative control
         Sera of unknown titers

   b. **Equipment**

      1) Test tubes (13 x 100 mm)
      2) Test tube supports
      3) Serologic pipettes:
         0.2-ml, graduated in hundredths
         1-ml, graduated in hundredths
         5-ml, graduated in tenths
         10-ml, graduated in tenths
      4) Centrifuge, International Size 2, with No. 250 head and No. 280 cups.
      5) Water bath (56°C)

6) Flasks (50-ml, 125-ml, 250-ml)
7) Spectrophotometer and cuvettes
8) pH meter
9) Interval timer

3. **Methods**

a. **Preparation of reagents and materials**

1) **Borate saline buffer, pH 8.2**

0.1 M Boric acid		50.0 ml
0.1 N NaOH		5.9 ml
NaCl		0.85 gm
Distilled water	to	100.0 ml

Determine the pH after all materials are thoroughly mixed. If it is not exactly pH 8.2, adjust with either NaOH or Boric acid.

2) **Polystyrene latex suspension (1% stock suspension)**

a) Mix well and dilute the commercial latex particle suspension to an estimated 1% total solids with borate saline buffer. Some commercial products, e.g., Difco 0.81μ, are already diluted and ready to use.

b) Filter the 1% suspension through Whatman No. 40 filter paper to eliminate any aggregated particles. The filtrate is the stock 1% suspension.

c) In order to standardize and check the density of the 1% stock suspension, measure the optical density of a 1:100 dilution of the 1% suspension (0.1 ml of 1% suspension + 9.9 ml of borate saline buffer, mix well) using a Coleman Junior spectrophotometer at a wavelength of 650 nm and 12 x 75-mm cuvettes. The optical density should read 0.28 ± 0.02. The 1% stock suspension may be stored at 4°C for several months.

d) If the O.D. reading is not 0.28 ± 0.02, calculate the dilution of the stock solution needed to obtain the desired 1% suspension:

$$\text{Final vol. of stock suspension} = \frac{(\text{Actual O.D. of test susp.}) \times (\text{Vol. of stock susp})}{\text{Desired O.D.}}$$

Example:

The O.D. of the 1:100 dilution reads 0.35 and you have 24 ml of the stock suspension:

$\frac{(0.35) \times (24)}{0.28}$ = 30.0 ml, **final vol.** of desired 1% suspension.

Therefore, you would dilute (adjust) the stock suspension by adding 6.0 ml of borate saline buffer to the 24 ml of stock suspension.

3) **Gamma globulin solution (0.5% stock solution)**

a) To 0.5 gm of lyophilized gamma globulin, add borate saline buffer in 10-ml portions, mixing each time saving the supernatant until a total of 100 ml is obtained and the gamma globulin is dissolved.

b) Before use, the gamma globulin solution should be stored for 24 hours at 4°C to make sure all has gone into solution.

c) The solution is good for several weeks at 4°C, if kept uncontaminated.

b. **Performance of the test**

1) Set up a rack of 13 x 100-mm test tubes as follows:

a) For each serum to be tested (including the known positive and negative control sera), set up a row of 9 tubes.

b) Include 1 tube for the antigen control.

2) To the first tube of each row, add 1.9 ml of borate saline buffer.

3) To the remaining 8 tubes of each row, and to the antigen control tube, add 1.0 ml of borate saline buffer.

4) Add 0.1 ml of each serum to be tested to the first tubes, mix, and transfer 1 ml to the second tube. Mix, and transfer 1 ml to the second tube. Mix, and transfer 1 ml each time until all dilutions are made. Discard 1 ml from the last (9th) tube.

5) Prepare a latex-gamma globulin mixture as follows:

a) For each serum to be tested, mix together 9.4 ml of borate saline buffer and 0.1 ml of the 1% latex suspension. To this, add 0.5 ml of the 0.5% gamma globulin solution. Mix thoroughly.

6) To all tubes in the test, including the antigen control, add 1 ml of the latex-gamma globulin mixture.

7) Shake the tubes thoroughly and incubate for 2 hours in a water bath at 56°C.

8) Centrifuge the tubes at 2300 rpm for 3 minutes and read the agglutination pattern with the naked eye.

4. **Precautions and Helpful Suggestions**

a. The test should be performed under rigidly standardized conditions in order to obtain reproducible titers. Wherever possible, maintain constancy of latex concentration, make and source of gamma globulin, time of incubation, and temperature of incubation.

b. In control and negative sera there is an occasional agglutination of particles after centrifugation but these are readily and smoothly resuspended by agitation of the tube. Occasionally the control tube will show a fine granulosity and this should be taken into consideration in determining the final titer of the serum tested.

c. To be sure of the sensitivity of this test under day-to-day variations, it is necessary to include one or more sera of known strength in routine work.

5. **Interpretation of Results**

Agglutination in dilution of 1:20 or greater is considered a positive test. Between 69.7 and 86.0% of sera from cases of spondylitis rheumatica with peripheral rheumatoid symptoms have been found to react with the "sensitized" latex antigen.

Positive reactions have also been found in certain other rheumatic and nonrheumatic diseases:

rheumatic fever cases	Approx. 10%
periarteritis nodosa	Approx. 3%
Osteoarthritis	Approx. 3%
Fibrositis	Approx. 3%
Syphilis and TB	Approx. 5-10%

False positives have occurred in approximately 4% of sera. Usually, the titers in rheumatoid arthritis sera are much higher than those found in sera from patients with other diseases. These titers usually range between 1:500 and 1:1000, but may go as high as 1:80,000.

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Commercial Rapid Tests for Rheumatoid Factor

A number of commercial diagnostic reagent suppliers are marketing serologic kits for the laboratory detection of rheumatoid factor. The majority of these kits utilize latex particles coated with gamma globulin as antigen in the test. The procedures are based on the same principles as those of the Singer-Plotz test. Since the protocols for the various kits are all different, directions for each should be followed and the interpretation made according to the manufacturer's printed brochures.

The Latex Agglutination (LA) Test for Cryptococcosis

by Leo Kaufman, Ph.D. and Sharon O. Blumer, M.S.

Introduction

Cryptococcosis, is a systemic mycosis caused by *Cryptococcus neoformans.* This pathogenic yeast gains access to the body through the respiratory tract; however, primary infections of the skin and mucous membranes may occur on rare occasions. Pulmonary infection is secondary in importance to cryptococcosis of the central nervous system. This serious form of the disease results from hematogenous dissemination of the fungus from the initial pulmonary lesion.

Although cases of primary cryptococcosis have been reported, 30-50% of the cases are associated with such debilitating diseases as lymphosarcoma, leukemia, Hodgkin's disease, sarcoidosis, diabetes, and lupus erythematosus. In addition other factors such as prolonged therapy with corticosteroids, antibiotics and cytotoxic agents tend to predispose persons to cryptococcal infection.

Application of cultural and histologic procedures to prove *C. neoformans* infection is frequently time consuming and inadequate. In addition, studies with India ink smears to detect the yeast in spinal fluid are frequently negative with cerebrospinal fluid (CSF) specimens from meningeal cases. Fortunately in recent years significant progress has been made in the development and application of reliable immunodiagnostic tests. Three tests in particular have received wide use: the latex agglutination (LA) test for *C. neoformans* antigen, the indirect fluorescent antibody test, and the tube agglutination test for *C. neoformans* antibodies. Depending upon the clinical stage of disease, the patient may be positive either for antigen or antibody or both. Diagnosis is best achieved through the concurrent use of these three tests. The LA test in addition to detecting antigen in serum and urine is particularly effective in diagnosing meningeal infections.

A properly performed and controlled positive LA test is diagnostic for cryptococcosis. The reagents are stable, the test is simple and rapid, and it is reactive to antigen circulating in the host early in the disease.

Principles

1. Hyperimmune rabbit anti-*C. neoformans* globulin can be adsorbed onto inert polystyrene latex particles.

2. Very small amounts of cryptococcal capsular polysaccharide present in the body fluids of an infected host react with the anti-*C. neoformans* globulin adsorbed onto the surface of the latex particles. This results in a visible agglutination of the latex particles.

3. The quantity of antigen in the patient's body fluids (i.e., serum, spinal fluid, urine, etc.) can be determined by adding a constant volume of latex anti-*C. neoformans* globulin mixture to serial dilutions of a clinical specimen. The antigen titer is an expression of the specimen dilution containing enough polysaccharide antigen to properly agglutinate the antibody coated particles.

4. Sera that contain rheumatoid factor may in some cases react with the latex-rabbit antiglobulin mixture. Such false positive reactions can be identified by the use of latex control reagent consisting of latex particles coated with normal rabbit globulin.

1. **Materials Required**

   a. **Reagents**

      1) Glycine buffered saline (GBS), pH 8.4.

      2) GBS, pH 8.4 containing 0.1% bovine serum albumin.

      3) Polystyrene latex particle suspension (Difco, 0.81μ)

      4) Rabbit anti-*C. neoformans* globulin adjusted to 4 gm% protein and demonstrating a tube agglutination titer of 1:1024 or greater.

      5) Rabbit normal globulin adjusted to 4 gm% protein

      6) Specimens and controls:

         Positive specimen for positive control
         Normal serum for negative control
         Serum positive for rheumatoid factor
         Unknown serum, cerebrospinal fluid, and/or urine

      7) 1N NaOH

   b. **Equipment**

      1) Test tubes (13 x 100 mm)

      2) Test tube racks

      3) Glass slides (2" x 3")

      4) Wax glass marking pencil

      5) Serological pipettes:

         0.5 ml graduated in hundredths
         1.0 ml graduated in hundredths
         5.0 ml graduated in tenths

      6) Water bath (56°C)

      7) Toothpicks or applicator sticks

      8) Graduates (glass stoppered), 10 ml

      9) Spectrophotometer (Coleman, Jr.) with round cuvettes (12 x 75 mm)

      10) Rotary shaker with platform

      11) pH meter

2. **Preparation of Reagents**

   a. *Glycine Buffered Saline (GBS), pH 8.4*

      Mix:

      NaCl ..... 9.0 gm
      Glycine ..... 7.5 gm
      Distilled water, q.s. to 1,000 ml
      Adjust to pH 8.4 with 1N NaOH

   b. *GBS, pH 8.4 With 0.1% Bovine Serum Albumin (GBS/BSA)*
      Add one gram of bovine serum albumin (Fraction V) per liter of GBS. Mix thoroughly.

   c. **Standardized Polystyrene Latex Suspension**

      1) To determine whether the latex suspension is of the correct concentration, thoroughly mix the commercial latex particle suspension, and dilute 1:100 with GBS (0.1 ml latex suspension + 9.9 ml GBS) using a glass stoppered 10 ml graduate.

      2) Measure the optical density of the 1:100 dilution of latex in 12 x 75 mm cuvettes using a Coleman, Jr. spectrophotometer at a wavelength of 650 nm. If the optical density is 0.3 $\pm$ 0.02, the undiluted latex suspension is properly standardized.

      3) If the optical density reading is not 0.3 $\pm$ 0.02, calculate the dilution of commercial latex suspension needed to obtain the desired standardized suspension.

$$\text{Final volume of Standardized suspension} = \frac{\text{Actual optical density of 1:100 dilution} \times \text{Volume of commercial latex}}{\text{Desired optical density}}$$

Example: The optical density of the 1:100 dilution reads 0.50 and you have 5.0 ml of the commercial latex suspension:

$$\frac{0.50 \times 5.0}{0.30} = 8.3 \text{ ml final volume of standardized suspension}$$

Therefore, you would obtain the 8.3 ml of standardized suspension by adding 3.3 ml of GBS to the 5.0 ml of commercial latex suspension. Repeat steps 2c, 1 and 2c, to verify that the correct concentration of latex particles has been attained.

d. Sensitized Latex

1) Dilutions of the rabbit anti-*C. neoformans* globulin are made in GBS as follows:

0.1 ml antiglobulin + 9.9 ml GBS = 1:100
1 ml, 1:100 dilution + 1 ml GBS = 1:200
1 ml, 1:100 dilution + 2 ml GBS = 1:300
1 ml, 1:100 dilution + 3 ml GBS = 1:400

One ml of each dilution of antiglobulin is mixed thoroughly with 1.0 ml of standardized latex suspension and allowed to stand at room temperature for 15 minutes.

2) Using the GBS/BSA, make serial twofold dilutions of the known positive specimen.

Set up a row of 8 tubes (13 x 100) and add 0.5 ml of GBS/BSA to each one. Add 0.5 ml of the positive control specimen to the first tube, mix and transfer 0.5 ml to the second tube. Continue mixing and transferring 0.5 ml through tube #8. Discard 0.5 ml from the last tube (#8).

3) Determine the optimally reactive dilution of rabbit anti-*C. neoformans* globulin needed for sensitization.

a) Test each dilution of known positive specimen against each latex preparation sensitized with the various dilutions of antiglobulin.

On 2 x 3 inch slides, mark off 6 one inch squares with a wax pencil. In a 1 inch square, mix 0.04 ml of the 1:2 dilution of positive specimen with 0.02 ml of the latex sensitized with 1:100 dilution of antiglobulin.

In other squares mix 0.04 ml of each of the remaining dilutions of specimen with 0.02 ml of the same reagent. Place slides on a rotary shaker platform and rotate at 125 ± 25 rpm for 5 minutes. Record as positive all dilutions of specimen showing agglutination equal to or greater than 2+ (i.e., small but definite clumps against a slightly cloudy background). Repeat this same procedure with the latex sensitized with the 1:200, 1:300, and 1:400 dilutions of antiglobulin.

b) The optimally sensitized latex preparation is that which shows 2+ agglutination with the highest dilution of positive specimen. This dilution of the positive specimen will serve as the 2+ positive control for screening and titering unknown specimens.

e. **Control Latex (LC)**

1) The control latex (LC) reagent is prepared by mixing equai volumes of normal rabbit globulin, **prepared at the same dilution as the antiglobulin**, and standardized latex suspension. In general a total volume of 2.0 ml LC will be sufficient for the test.

Example: a) Optimal dilution of antiglobulin = 1:200, so the normal rabbit globulin is likewise diluted 1:200.

b) Mix 1.0 ml of normal rabbit globulin (1:200) and 1.0 ml of standardized 1% latex suspension.

3. **Performance of Test**

a. Inactivate all serum and cerebrospinal fluid specimens at 56°C in a water bath for 30 minutes. Urine specimens should be inactivated by boiling for 10 minutes.

b. Mark off a 2 x 3 inch slide into 6 one inch sections using a wax pencil.

c. In a single one inch square mix 0.04 ml of unknown undiluted specimen, with 0.02 ml latex reagent.

d. On the same slide, in other squares, mix additional unknown specimens, plus a positive control (2+) specimen and a negative control (normal serum), as above. Place slide on rotary shaker platform and rotate at 100-150 rpm for 5 minutes.

e. Record as positive only those specimens that show an agglutination equal to or greater than the 2+ positive control.

f. In the same manner (steps b - e) test all the positive serum specimens with LC reagent to rule out false positives due to rheumatoid factor. Use the positive rheumatoid factor serum as the positive control for LC reagent.

g. Positive undiluted cerebrospinal fluid and urine specimens and all sera positive with latex reagent and negative with LC should be titered.

1) Set up a row of 4 tubes (13 x 100 mm) for each specimen to be titered. Label tubes.

2) To the tubes of each row, add 0.5 ml of GBS/BSA as the diluent.

3) Add 0.5 ml of each specimen to be tested to the first tubes, mix, and transfer 0.5 ml to the second tube. Continue mixing and transferring 0.5 ml through tube 4. **DO NOT** discard 0.5 ml from the 4th tube.

4) On a marked slide mix 0.04 ml of each dilution of each specimen with 0.02 ml latex reagent and rotate as above. One slide will be sufficient to test 4 dilutions of each positive specimen plus positive and negative controls.

5) The titer of a positive specimen is the highest dilution that shows an agglutination equivalent to the 2+ positive control. If the reaction of the 1:16 dilution (tube 4) is stronger than 2+, continue diluting the specimen through 4 more tubes (1:32 - 1:256) and repeat the testing procedure until a titer is determined.

4. **Precautions and Suggestions**

a. The LA test should always be performed under carefully standardized conditions. Maintain constancy of latex concentration, volume of reactants, reaction time, rotating speed and degree of agglutination of the 2+ positive control.

b. Both the latex and the LC reagents are stable at 4°C for 12 months or more. Freezing, however, will destroy these reagents. It is always necessary to include a negative control in each run to verify the stability of the latex reagents. If the negative control fails to show a homogenous, milky suspension with no agglutination when reacted with the LC and latex reagents, then the test is not valid.

c. Likewise, the test is invalid if the positive control shows agglutination greater or less than the established 2+ degree or the latex reagent fails to yield a given titer with a known positive specimen previously tested.

d. It is advisable to centrifuge or filter all specimens that contain any visible amount of particulate matter. All specimens not to be cultured should also be preserved with merthiolate (1:10,000).

5. **Interpretation of Test Results**

a. The LA test for cryptoccal antigen appears to have diagnostic as well as prognostic value in that increasing titers reflect progressive disease, while declining titers indicate response to chemotherapy and progressive recovery. Cryptococcal antigen in body fluids of the untreated patient indicates active disease; however, in some instances LA titers remain positive at a low level for an indefinite period of time during which the fungus is no longer viable in the treated patient.

b. LA tests, in which serum specimens react with both latex and LC reagents, should be considered equivocal. However, since it is possible that cryptococcosis and arthritic conditions can occur concomitantly, titers with both latex reagent and LC should be determined.

1) If a fourfold or greater titer occurs with the latex reagent, this may suggest that cryptococcosis is a real possibility, and additional specimens should be examined for titer change.

2) Reactions of less than 2+ in undiluted spinal fluids should be regarded as highly suggestive of cryptococcosis, and additional specimens should be examined for evidence of increasing titer.

c. The properly controlled LA test appears to be 100% specific. Hundreds of serum and cerebrospinal fluid specimens from patients with a variety of bacterial, viral, and fungal infections other than cryptococcosis have been found to be non-reactive. The LA test has generally been shown to be a reliable and valuable adjunct to the diagnosis of cryptococcosis.

d. A negative reaction should not exclude a diagnosis of cryptococcosis, particularly if only a single specimen has been tested and the patient shows symptoms consistent with those of cryptococcosis.

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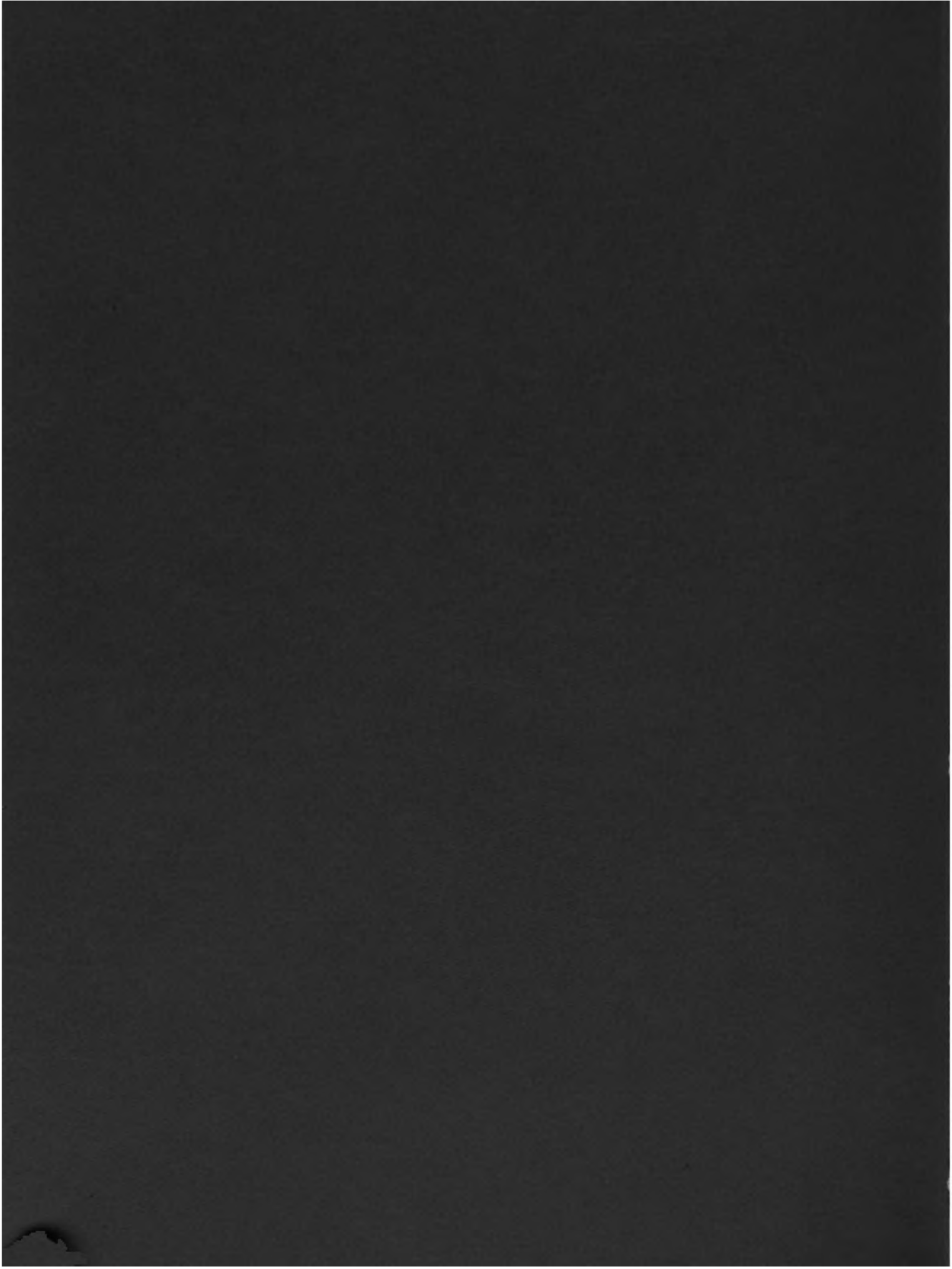

Bentonite Flocculation Test (BFT) for Trichinosis

Infection of man by *Trichinella spiralis* can occur when raw or insufficiently cooked pork is ingested. Hogs fed on uncooked garbage and meat scraps from slaughter houses and markets are likely to be infected, and, when slaughtered for human consumption, pass the encapsulated larvae to man. The larvae mature in the small intestine and produce live young. These young larvae enter the blood stream and are circulated throughout the system, where they burrow into muscle fibers.

In trichinosis, a distinction should be made between zoological and clinical infection. Only a small percentage of infected persons have sufficient parasites to produce clinical trichinosis. For these, prognosis is ordinarily good, though severe infections may result in death. There are three clinical phases of the disease corresponding to the following periods: 1) intestinal invasion by adult worms, 2) migration of the larvae, and 3) encystment and repair. Fever will remain consistent as the illness runs its course, and diarrhea, muscle pain, and weakness are common symptoms. The presence of adult and larval Trichinae produces an immunity demonstrated by the production of antibodies.

Since symptoms of trichinosis resemble those of many other diseases, definitive diagnosis often depends upon the several biologic and laboratory tests that are available. Muscle biopsy may be performed during the third or fourth week of infection, and is used for confirmation of clinical diagnosis and serologic findings.

The skin test, complement-fixation test and precipitin test are usually negative early in the infection. A negative followed by a positive test or rising titer is diagnostic. The rapid flocculation test is as sensitive as complement-fixation, and has the added advantage of being quick. The reagents are stable, the test is simple, and it is reactive to antibodies in serum early in the illness.

1. **Principles**

   The bentonite flocculation test for trichinosis is based on the following principles:

   a. Very small amounts of antibody can elicit a visible reaction with antigen adsorbed onto inert particles.

   b. Bentonite particles adsorb soluble *T. spiralis* antigen and form a stable lattice work or flocculate in the presence of antibody to *T. spiralis.*

   c. The amount of trichina antibody present in the patient's serum can be determined by combining serial dilutions of serum with a constant amount of a standardized suspension of antigen-coated bentonite particles. The antibody titer is an expression of the serum dilution containing just enough antibody to agglutinate 75-100% of all particles.

2. **Materials**

   a. **Reagents**

      1) Stock bentonite suspension
      2) Trichina extract
      3) Stock antigen - adsorbed bentonite suspension
      4) Thionin solution
      5) 0.85% NaCl
      6) Test antigen - adsorbed bentonite suspension
      7) Tween 80 preparation
      8) Normal serum for negative control
      9) Positive control serum with high titer
      10) Positive control serum with low titer
      11) Patient's serum

b. **Equipment**

1) Screw cap flask - 50 ml
2) Graduated cylinder - 25 ml
3) Centrifuge tubes - 15 ml
4) Wax ring slides
5) Dropping pipettes - should deliver 60-80 drops per ml
6) Boerner type rotating machine
7) Microscope with 10X objective and 12X ocular
8) Pipettes, 1 ml, 5 ml
9) Small tubes (13 x 100) for making dilutions
10) Water bath (56℃)
11) Centrifuge tubes - 50 ml

3. **Methods**

a. **Preparation of reagents and materials**

1) Stock bentonite suspension

It is important to have a suspension of particles of uniform size. It is best prepared by fractional centrifugation: first at 500 X G to remove large particles, followed by 750 X G to sediment the desires sizes. All glassware **must** be clean.

a) Suspend 0.5 gm of bentonite in 100 ml of glass-distilled water.

b) Homogenize in Waring Blender, or equivalent, for 1 minute.

c) Transfer bentonite suspension to a 500 ml glass-stoppered graduate and add glass-distilled water to make 500 ml. Shake thoroughly.

d) Centrifuge in 50 ml centrifuge tubes for 15 minutes at 500 X G.

e) Pour off and save supernate; discard sediment.

f) Centrifuge supernate at 750 X G for 15 minutes.

g) Pour off and discard supernate.

h) Resuspend sediment in 100 ml distilled water and homogenize in blender for 1 minute.

This "stock" bentonite will normally remain stable for as long as 4 months without losing its adsorptive properties if stored at 4℃.

2) Thionin solution

Thionin	0.1 gm
Distilled water	100 ml

3) Tween 80 preparation

Tween 80	0.5 ml
Distilled water	99.5 ml

Prepare immediately prior to use.

4) Stock antigen - adsorbed bentonite suspension

a) Shake stock bentonite solution well and transfer 5 ml to a flask containing 2.5 ml of trichina extract. Mix and store at 4℃ overnight. Antigen is adsorbed onto the particles in this step.

b) On the following day, add 1.25 ml of 0.1% thionin solution and allow to stand 1 hour at 4℃ to insure complete adsorption of the dye to the coated particles.

This "stock antigen" may be used at least 3 months after preparation if kept at 4℃.

5) Test antigen - adsorbed bentonite suspension

a) Shake stock antigen suspension well and transfer 4 mls to a 15 ml conical centrifuge tube.

b) Add sufficient 0.85% NaCl to the tube to fill it and centrifuge at 1300 x G for 5 minutes.

c) Pour off supernate, resuspend the sediment in 15 mls 0.85% NaCl and re-centrifuge.

d) Pour off supernate and resuspend the sedimented particles in 2 ml of saline.

b. Standardization of Test Antigen - Adsorbed Bentonite Suspension

1) Check the particles in saline for the presence of non-specific flocculation. No more than 50% flocculation should occur in the saline.

a) Pipette 0.1 ml saline into a well on the glass slide.

b) Mix antigen thoroughly and with a dropping pipette, add a drop of antigen to the well.

c) Rotate the slide on the rotating apparatus at 100-120 rotations per minute for 15 minutes, or for a shorter time if too much agglutination occurs in the saline.

d) Examine the slide with a microscope. If too much agglutination occurs in the saline control, then the antigen must be adjusted with a freshly prepared 0.5% Tween 80 solution.

2) Add 0.05 ml Tween 80 preparation to the antigen suspension. Shake well and again test with saline as described under step 1.

If too much agglutination occurs again, then add an additional 0.05 ml of Tween 80 to the antigen. Repeat until the antigen is adjusted.

3) After the antigen is adjusted with Tween 80, test it against a negative serum and 2 positive sera, 1 with a high and 1 with a low titer.

All 3 sera are run at 3 dilutions. The negative serum is run at 1:5, 1:10, and 1:20. The positive sera are run so that the middle dilution corresponds with the titer which has been previously determined.

4) Pipette 0.1 ml of each serum dilution onto a slide and drop the antigen into the wells.

5) Rotate the slides for 15 minutes at 100-120 rotations per minute.

6) Examine slides with dissecting microscope for the presence of 3-4+ flocculation at the expected titer.

c. Performance of Routine Bentonite Flocculation Test

1) Inactivate the sera for 30 minutes at 56°C.

2) Make a 1:5, 1:10, and 1:20 dilution of unknown sera with saline. These dilutions are screened first.

a) Add 0.2 ml serum to 0.8 ml saline. This gives the 1:5 dilution. Make serial dilutions in 0.5 ml volumes to obtain the 1:10 and 1:20 dilutions.

b) Since positive sera are diluted further until negative results are obtained, do not discard the 1:20 dilution.

3) Pipette 0.1 ml serum dilutions onto ringed slides. Start with highest dilution and use the same pipette for each serum.

4) Mix the standardized antigen well. Add 1 drop of it to each ring.

5) Rotate slides for 15 minutes at 100-120 rotations per minute.

6) Examine slides with microscope.

7) Sera which are positive at 1:20 should be further diluted until a negative reaction is obtained.

d. Reading the Test

4+ All particles are agglutinated.
3+ 75% of particles agglutinated.
2+ 50% of particles agglutinated.
1+ 25% of particles agglutinated.

A 3+ and 4+ agglutination is considered positive.
A 2+ and 1+ agglutination is considered negative.

Note: For each series of tests, a saline control and negative and positive serum controls should be made.

4. Precautions and Helpful Suggestions

a. Use *only* glass distilled water in the preparation of the stock bentonite suspension. Any non-volatile material remaining in the water can cause non-specific flocculation or inhibit the bonding of antigen to particles.

b. Avoid using contaminated bentonite. Contamination causes non-specificity and poor adsorption of antigen to particles.

c. Serum which is contaminated is not satisfactory for use. That which must be shipped should have 1:10,000 aqueous merthiolate added.

d. Serum having excessive lipid content should be defatted before testing. The lipids can coat the particles and completely inhibit flocculation.

e. Well-slides are not satisfactory for use in this test.

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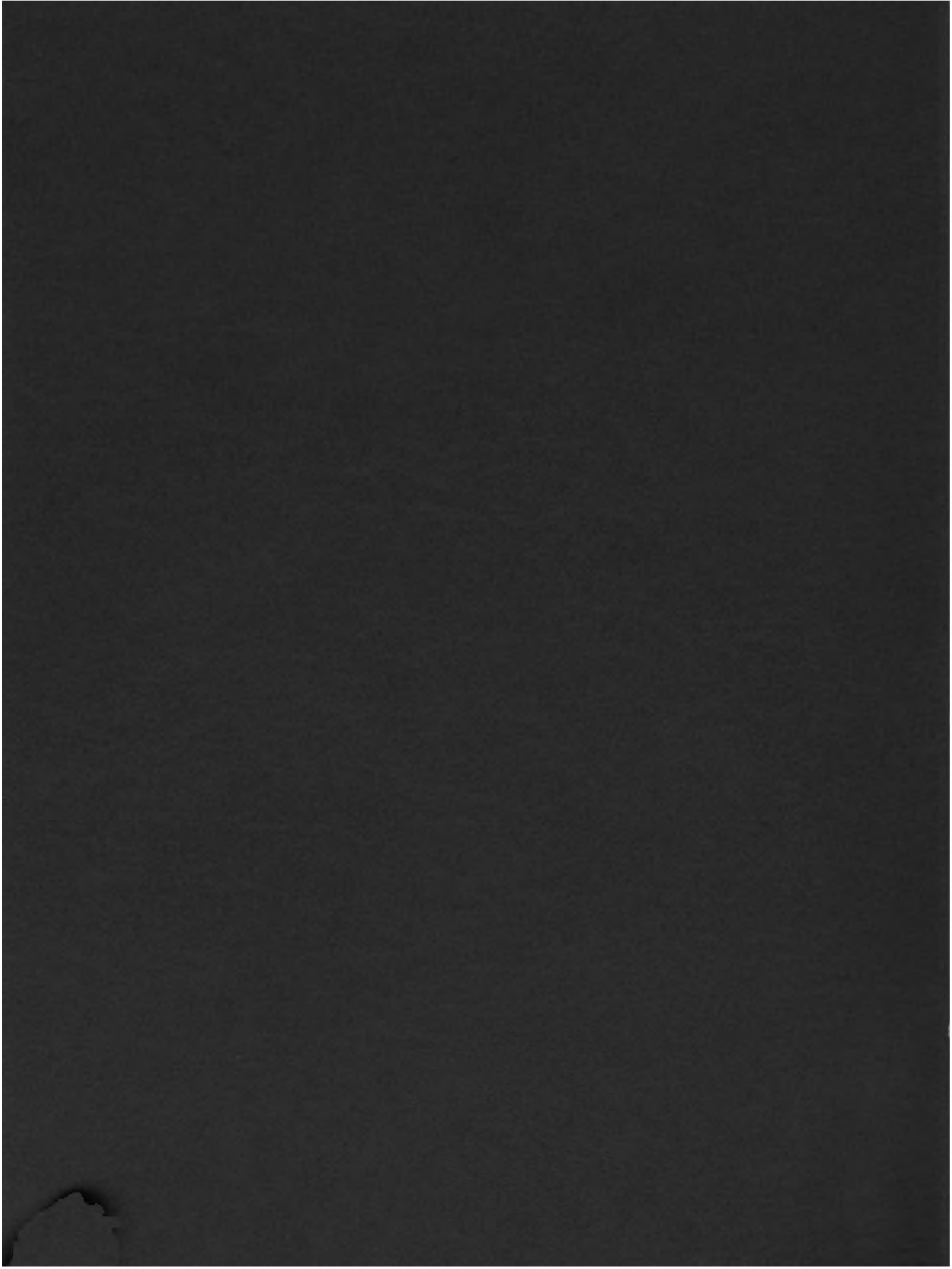


Agar-Gel Immunodiffusion Test for Histoplasmosis

Histoplasmosis is caused by a yeast-like fungus, *Histoplasma capsulatum*, and is generally characterized by emaciation, irregular fever, leukopenia, anemia and splenomegaly. Three clinical types of histoplasmosis have been recognized: 1) disseminated histoplasmosis, a generalized infection involving organs containing reticulo-endothelial cells, i.e., spleen, liver, and lymph nodes. This type of histoplasmosis is the least common, but occurs at all ages with an over-all mortality of 75%; 2) the acute pulmonary form, exhibiting the principle symptoms of fever, cough, chest pains, chills, sweat, and dyspnea. This form of histoplasmosis occurs over a wide age range, but is usually benign, having a duration of 19 days to 3 months; 3) the chronic pulmonary form, occurring mostly in adults. This type of histoplasmosis has an average duration of about five years in fatal cases and is fatal in perhaps less than 20% of all cases.

H. capsulatum exists in soils, particularly when they are enriched as in chicken houses and rat burrows. Histoplasmosis apparently is not transferred from person to person, but is acquired exogenously. Exposure of human beings to dust contaminated with spores (the infectious form of *H. capaulatum*) is the mode of infection.

Due to the varying clinical manifestations of histoplasmosis, laboratory procedures are essential to diagnosis. Dermal sensitivity to histoplasmin persists for years after recovery and may be of little use in an endemic area due to the high incidence of positive but otherwise healthy reactors. The skin test is also complicated by frequent cross-reactions with other mycoses, such as blastomycosis, and coccidioidomycosis. Culturing of *H. capsulatum* is probably the most conclusive laboratory procedure, but is not always easy to accomplish. The Agar-Gel Precipitin test in conjunction with other serologic procedure, e.g., the Complement Fixation test, is of considerable value in the diagnosis and prognosis of histoplasmosis. It can be performed with relative ease, and in most cases will distinguish hypersensitivity and chronic infection from an active case of histoplasmosis. It is especially useful when the complement fixation test is negative or the patient's serum is anticomplementary.

1. **Principles**

   The agar-gel precipitin test for histoplasmosis is based on the following principles:

   a. Homologous antigen and antibody, diffusing toward each other through a semisolid medium, will mix at some point in optimal or near optimal proportions to form a visible band of precipitate.

   b. *Histoplasma* antibodies in a patient's serum will precipitate one or more antigens present in a histoplasmin preparation.

   c. *Histoplasma* antibodies in a patient's serum can be identified by allowing the patient's serum and the known histoplasmin antigen to diffuse toward each other through a semisolid medium and then comparing the resulting bands of precipitation with those of a reference antiserum.

2. **Materials**

   a. **Reagents**

      1) Agar medium
      2) Histoplasmin antigen (prepared from mycelial phase of *H. capsulatum*)
      3) Sera
         a) Positive (containing H and M precipitins)
         b) Negative
         c) Patient's

b. **Equipment**

1) 2" x 3" glass slides, pre-cleaned
2) Pipettes, Pasteur
3) Serologic pipettes, 10 ml
4) Applicators, cotton tipped
5) Pattern for cutting wells in agar medium
6) Die (3 mm) for cutting wells in agar medium
7) Reading box containing fluorescent light source*

3. **Methods**

a. **Preparation of Reagents and Materials**

1) 1% Agar Medium

a) 0.9 gm Sodium chloride
b) 1.0 gm Purified Agar (Noble's Special Agar or equivalent)
c) 0.4 gm Sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$)
d) 0.25 ml Phenol (88%)
e) 7.5 gm Glycine
f) Distilled H_2O ... q.s. to 100 ml (Heat to dissolve)

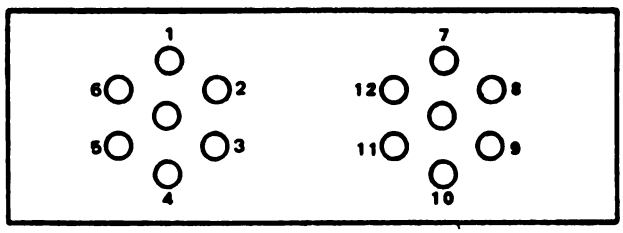
Before use, autoclave at 15 lbs pressure for 10 minutes. Final pH 6.7 - 6.8.

2) 0.2% Agar dilution

Add 2 ml of melted 1% agar to 8 mls of distilled water (Heated to 70° to 80°C.)

b. **Performance of Test**

1) Swab hot 0.2% agar onto two clean 2" x 3" slides and place in 37°C incubator for 30 minutes to dry.
2) Pipette 7 ml of 1% agar onto each of the pre-coated slides. (Temperature of agar should be 60°-65°C.)
3) Allow agar to harden.
4) Cut two patterns in agar on each slide as shown below:



The six outer wells should be 4 mm from each other and 4 mm from the center well. Each well is 3 mm in diameter.

To remove agar from wells, use pasteur pipettes connected to light suction.

*A 22 watt 8 inch circular fluorescent tube mounted two inches below the top of the reading box. The background beneath the light source should be a flat black finish.

5) Using Pasteur pipettes, fill wells with reagents as follows:

Slide #1

Well	Reagents
Center	Histoplasmin
1 and 4	Known Positive Serum
7 and 10	Known Positive Serum
2 and 8	Known Negative Serum
3 and 9	Student's Serum
5 and 11	#1 Unknown Serum
6 and 12	#2 Unknown Serum

Slide #2

Well	Reagents
Center	Histoplasmin
1 and 4	Known Positive Serum
7 and 10	Known Positive Serum
2 and 8	Known Negative Serum
3 and 9	#3 Unknown Serum
5 and 11	#4 Unknown Serum
6 and 12	#5 Unknown Serum

6) Incubate at room temperature (25℃) in a moist chamber.

7) Read slides at 24 and 48 hours.

8) Look for lines of precipitation. To facilitate reading the reactions, a view box containing a fluorescent bulb above a black background may be used.

4. Precautions and Helpful Suggestions

a. Be sure that the temperature of the agar medium is 60°-65℃.

b. The top of each slide should be marked so that the wells can be identified. Scratch the glass slide or label in some manner that will not be affected by moisture. Label the slide, not the petri dish containing the slide.

c. When removing agar from wells, use light vacuum. Do not touch the bottom of the well with the pipette when removing agar.

d. Completely fill the wells with reagents, but **DO NOT** overfill.

e. Agar medium may be added to slides and the slides stored for later use, provided they are kept in a moist chamber at 4℃. (This is conveniently accomplished by placing the slides in petri dishes which are placed in a can containing a wet paper towel or equivalent.) The prepared slides should be kept at 4℃ and used within two weeks.

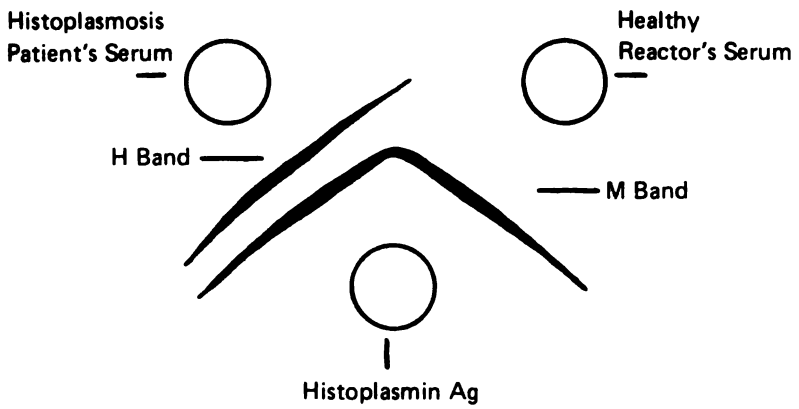
f. Agar medium may be prepared, autoclaved, and stored at 4℃ for later use. However, once the agar medium has been remelted it must be used or discarded. Storing agar in small amounts eliminates unnecessary waste of media.

5. Interpretation of Results

Appearance of one or more thin opaque lines or bands of precipitation between the antigen and serum wells is a positive reaction. The "M" band generally occurs nearer the antigen well and usually indicates hypersensitivity or chronic infection. The "H" band generally occurs nearer the antiserum (patient's serum) well and is usually due to active histoplasmosis. An "H" band rarely occurs in the absence of an "M" band.

The precipitin reactions will usually occur in 16-18 hours and should be read within two days.

On the next page is a pattern of typical reactions of (1) active histoplasmosis and (2) a positive but otherwise healthy reactor (dermal sensitivity or chronic infection).



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RADIAL IMMUNODIFFUSION TEST FOR THE QUANTITATION OF IMMUNOGLOBULINS

The radial diffusion type of precipitin reaction in agar has been found to be a useful means for estimating the quantity of immunoglobulins present in various body fluids. This procedure is a form of single diffusion based on the principles of the immune precipitin reaction between soluble antigen and its homologous antibody. It is performed by incorporating one of the two immune reactants (usually antibody) uniformly throughout a layer of agar gel, and then introducing the other reactant (usually antigen) into wells punched in the gel. The antigen (i.e., immunoglobulin) diffuses radially out of the well into the surrounding gel-antibody mixture and a visible disc (or ring) of precipitate forms where the antigen and antibody have reacted.

When material containing an immunoglobulin of unknown concentration diffuses radially from a well in a uniformly thin layer of antibody-containing agar, a systematic, quantitative relationship develops between certain dimensions of the precipitate disc and the concentration of the immunoglobulin. This relation is **linear** when the area of precipitate disc is plotted against concentration of antigen, provided that diffusion is allowed to proceed until all antigen has combined. The relation is **curvilinear** (linear during a certain transient instant in time) when the logarithm of antigen concentration is plotted against the diameter of the precipitate disc. The latter relationship is often used by laboratories because results of the assay can be obtained quicker. Using either method of plotting, the quantity of immunoglobulin present in the material of unknown concentration can be estimated by comparison with results obtained from a standard immunoglobulin preparation of known concentration.

Application of this procedure for the assay of immunoglobulins provides data of clinical importance in many diseases. Among these are pathologic states associated with 1) generalized **reduction** of one, two, three, or more of the immunoglobulin classes, 2) generalized **elevation** of one, two, three, or more of the immunoglobulin classes, and 3) several varieties of abnormalities as seen in multiple myeloma, Waldenstrom's macroglobulinemia, and various related protein disturbances. Also with this procedure, immune globulin replacement therapy may be monitored and controlled.

I. Materials

A. Reagents:

1. Phosphate Buffer
2. Special Noble Agar
3. Bovalbumin saline
4. Thiazine Red R Stain
5. Sera:
   a. Secondary Standard (Undiluted + 3 dilutions)
   b. Internal Controls (High level and low level)
   c. Patients' Test Sera
6. Antisera:
   a. Anti-IgG γ chain specific
   b. Anti-IgA α chain specific
   c. Anti-IgM μ chain specific
   d. Anti-IgD δ chain specific
   e. Anti-Kappa κ chain specific
   f. Anti-lambda λ chain specific

B. **Equipment:**

1. 3¼" x 4" glass slides, pre-cleaned
2. Serologic pipettes, 5 ml, 10 ml
3. Graduated cylinders, 100 ml
4. Erlenmeyer flasks, 250 ml
5. Staining dishes
6. Capillary tubes (1.2 mm - 1.5 mm O.D.)
7. Test tubes, 50 ml
8. Suction flask with rubber tubing
9. Waterbath, 56℃
10. Refrigerator, 4℃
11. Torsion balance
12. Interval timer
13. Tripod with asbestos pad (or hot plate)
14. Bunsen burner
15. Humidity chamber
16. Measuring magnifier (graduated to 0.1 mm)
17. Template for cutting wells
18. Well punch (3mm diameter)
19. Thermometer
20. Semi-log paper, 2-cycle
21. Filter paper (Whatman #1)
22. Shallow pan
23. Test tube rack
24. Cotton-tipped applicator sticks
25. Marking pencil

II. Preparation of Reagents

A. Phosphate Buffer pH 8.0

Chemicals needed:

KH_2PO_4

K_2HPO_4

NaN_3

Distilled Water

1N NaOH

1N HCl

1. Prepare Stock Solutions (10x concentrated) as follows:

   Solution A:

   a. Add 950 ml distilled water to a 1-liter volumetric flask fitted with a glass stopper.

   b. Add 52.26 gm K_2HPO_4 to the distilled water and mix thoroughly to dissolve.

   c. Add distilled water q.s. to 1-liter, mix again, and set aside.

   Solution B:

   a. Add 90 ml distilled water to a 100 ml volumetric flask fitted with a glass stopper.

   b. Add 4.08 gm KH_2PO_4 to the distilled water and mix thoroughly to dissolve.

   c. Add distilled water q.s. to 100 ml, mix again, and set aside.

2. Prepare Stock Phosphate Buffer (10x concentrated), pH 8.0, as follows:

   a. Add 950 ml of **Solution A** to a 1-liter mixing cylinder.

   b. Add 50 ml of **Solution B.**

c. Add 6.5 gm NaN_3 to the mixture and mix thoroughly to dissolve.

d. Check the pH with a pre-standardized pH meter. The pH should be 8.0 ± 0.05. If the pH is below 7.95, adjust with 1N NaOH. If the pH is above 8,05, adjust with 1N HCl.

e. Store this Stock Phosphate Buffer in a tightly closed bottle at 4°C. It may be stored for several weeks but check the pH often. Discard at the first sign of contamination or change in pH.

3. Prepare "Working" Phosphate Buffer, 0.03 M, pH 8.0 as follows:

a. Determine the volume of working buffer needed for the day's activity.

b. Prepare the needed volume by mixing together 1 part **Stock Phospate Buffer** and 9 parts distilled water.

c. Discard any remaining "Working" Phosphate Buffer at the end of the day.

B. 3% Special Noble Agar in Phosphate Buffer

Chemicals needed:

Special Noble Agar
"Working" Phosphate Buffer

1. Add 97 ml "Working" Phosphate buffer to a 250 ml Erlenmeyer flask.
2. Weigh out and add 3.0 gm Special Noble Agar to the Buffer.
3. Place the flask on an asbestos pad over a Bunsen burner and heat with agitation until agar is completely dissolved (as indicated by lack of turbidity). Keep loosely covered during heating to retard evaporation.
4. Loosely stopper the flask, transfer to a 56°C waterbath, and allow to cool to that temperature before using.*

C. 2% Acetic Acid Solution

Mix:

Glacial Acetic Acid	10 ml
Distilled water	490 ml

D. 1% Acetic Acid Solution

Mix:

Glacial Acetic Acid	10 ml
Distilled water	990 ml

E. 1% Acetic Acid - 1% Glycerol Solution

Mix:

Glacial Acetic Acid	5 ml
Glycerol	5 ml
Distilled water	490 ml

F. Thiazine Red R Staining Solution

Mix:

Thiazine Red R Stain	1.5 gm
1% Acetic Acid	498.5 ml

*Note: Larger volumes of 3% Special Noble agar can be made, allowed to gel in convenient aliquots, and stored for several weeks at 4°C. When needed, the agar is remelted in a boiling waterbath.

G. **1% Bovalbumin Saline Solution**

Mix:

Bovine Albumin (Fraction V)	2.0 gm
NaCl	1.7 gm
Distilled water	q.s to 200.0 ml

Sterilize by filtration through a Millipore membrane, 0.22 μ pore size and store at 4°C.

H. **Secondary Standard Serum**

See Quality Control Section, page 88.

I. **Internal Control Sera**

See Quality Control Section, page 90.

III. Preparation of Agar Slides

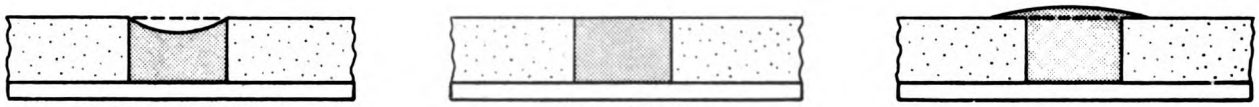
1. Estimate the volume of 3% Special Noble Agar needed for the test. Each 11 sera (or smaller fraction) to be assayed for a given class of immunoglobulin requires 8 ml of 3% Special Noble Agar plus a small amount extra for pipetting wastage.
2. Prepare the 3% Special Noble Agar solution as indicated on page F-11 and allow to cool to 56°C.
3. **Prepare appropriate dilutions of monovalent anti-immunoglobulin antisera*** in phosphate buffer for subsequent mixing with the agar solution. The volume of antiserum dilution needed is 8 ml for each 11 sera (or smaller fraction) to be tested plus a small amount extra for pipetting wastage. Prepare the dilutions in 50 ml test tubes and place in the 56°C waterbath for several minutes.
4. Pipette into the pre-warmed antiserum dilution an equal volume of 3% Special Noble Agar and mix well.
5. Determine the number of 3¼" x 4" pre-coated** glass slides required. One plate is required for each 11 sera (or smaller fraction) to be tested for each immunoglobulin class.
6. Place the pre-coated glass slides on a flat, level surface with the coated side facing upward.
7. Working rapidly, decant or pipette the agar-antiserum mixture onto the slides in the amount of 16 ml per slide. Guide the agar over the entire slide surface with a pipette so that a smooth, flat agar layer is obtained before the gelling process begins. While agar is still fluid, remove any bubbles by playing the flame of a Bunsen burner over the agar surface or by touching the bubbles with a hot needle.
8. Cover the slides and allow agar to solidify for approximately 20 minutes at room temperature.

*Note: See Appendix, page 96 for method of determining appropriate antiserum dilutions.

**Note: See Appendix, page 95 for method of pre-coating glass slides.

IV. Performance of the Test

1. Using a template and punch, cut 35 wells (3 mm diameter) 1.2 cm apart (5 rows of 7 wells each) in each of the agar slides. See Figure 1, page 84.
2. Remove the agar plugs from the wells by suction with a Pasteur pipette.
3. Label a number of 2-dram vials corresponding to the patient's sera to be tested.
4. With a 0.2 ml pipette, add 0.1 ml of phosphate buffer to each of the labelled vials.
5. Using a different 0.2 ml pipette for each serum, add 0.1 ml of each patient's serum to its corresponding vial containing 0.1 ml phosphate buffer. This makes a 1:2 dilution of serum. Mix thoroughly by agitation.
6. Align known standard serum dilutions, internal control sera, and all patient's sera in the order to be added to wells.
7. With capillary tubes approximately 1.5 mm O.D., add specimens to slides as follows: fill wells 1 through 4 and 19 through 22 with each of the 4 known standard serum dilutions **in duplicate**, as shown in Figure 1.



Underfilled Well | Properly Filled Well | Overfilled Well

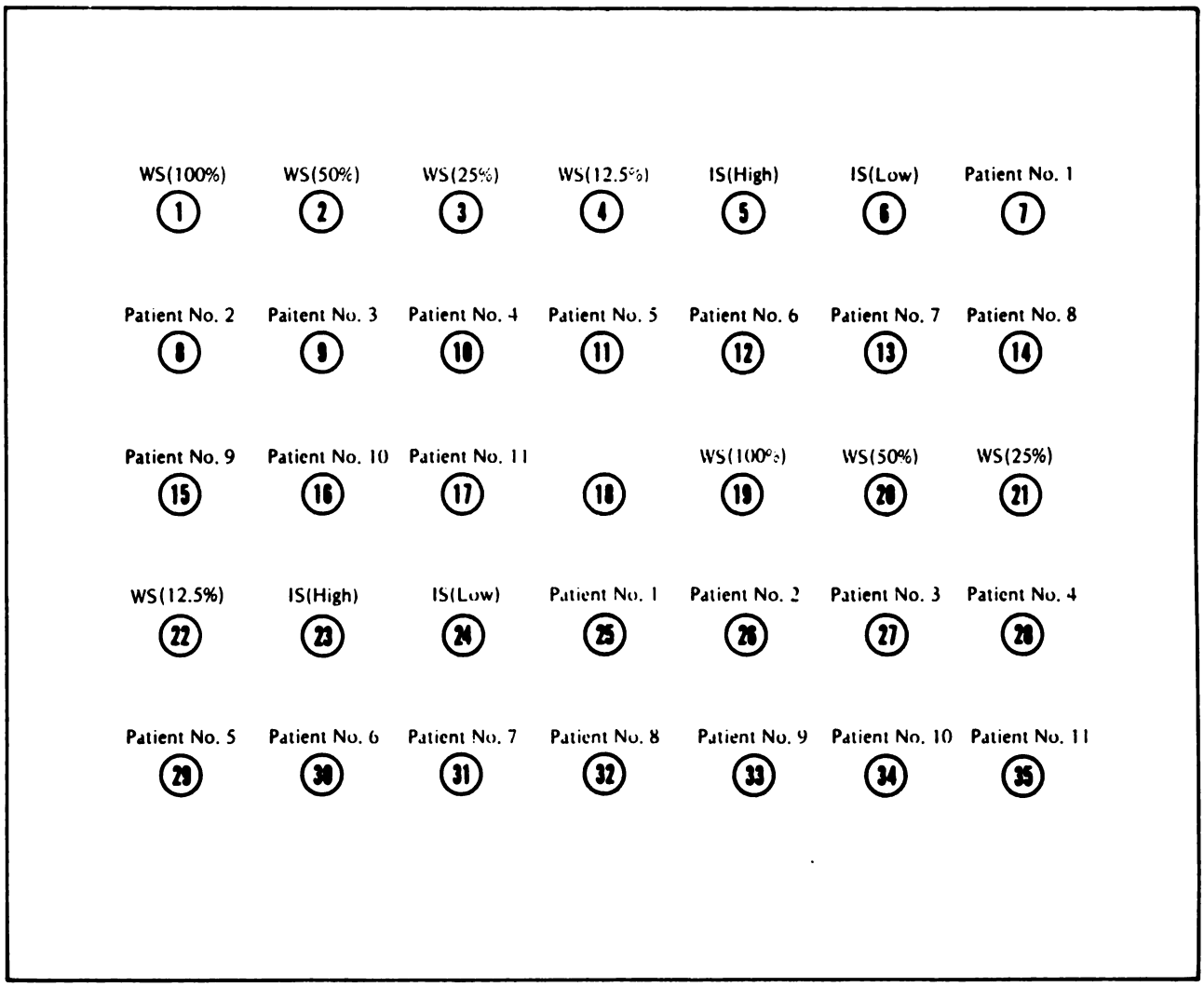
8. Fill wells 5, 6, 23, and 24 with the 2 internal control sera **in duplicate** as shown in Figure 1.
9. Fill wells 7 through 17 and 25 through 35 with patient's sera **in duplicate** as shown in Figure 1.
10. After adding all specimens to the slides, incubate in humid chambers for the times and temperatures designated in Table 1.

Table 1

Immunoglobulin Class	Incubation* Period	Incubation Temperature
IgG	16 – 20 hours	4°C
IgA	16 – 20 hours	4°C
IgM	24 – 36 hours	Room Temperature
IgD	20 – 24 hours	Room Temperature
Kappa	16 – 20 hours	4°C
Lambda	16 – 20 hours	4°C

*These time periods were chosen to approximate a linear relation between the logarithms of antigen concentrations and the diameters of the precipitin discs at the temperatures indicated.

FIGURE 1 LOCATION OF SPECIMENS ON PLATE



WS=Working Standard
IS=Internal Standard

11. After incubation, all slides may be read immediately, or may be further processed and read after staining. With some antisera, IgD slides must be stained for the precipitate to become visible.

If you elect to further process all the slides and stain them, see method of staining in Appendix, page 95.

V. Reading of Slides and Calculation of Results

1. Turn the slide upside down, place a measuring magnifier (graduated to 0.1 mm) on the glass side (not the agar) and position the magnifier over well #1. Ascertain the diameter of the precipitate disc around well #1 and record. If the precipitate disc is oval, rather than round, measure the diameter along the long axis and the diameter along the short axis and record the average of these measurements.
2. Move the magnifier to well #2 and measure the diameter of its corresponding precipitate disc. Record the result. Determine diameters for all of the precipitate discs in turn and record results. Compute averages for all duplicate determinations and record.
3. Obtain a sheet of **2-cycle semi-logarithmic drawing paper** and label the vertical (logarithmic) axis "International Units IgG (IgA, IgM, IgD, etc.) per Milliliter" and the horizontal (arithmetic) axis "Disc Diameter in Millimeters."
4. Examine the data obtained in steps 1 and 2 and determine the smallest and largest disc diameters obtained with the **secondary** standard serum dilutions. Mark off the horizontal axis on the semilogarithmic paper with a range of numbers which includes the smallest and largest disc diameters. Attempt to establish as great a spread as possible between the smallest and largest disc diameters on the horizontal axis so that maximum precision can be obtained.
5. Mark off the vertical axis with numbers corresponding to the range of dilutions of the secondary standard serum used.
6. Construct a reference line on the semi-logarithmic paper relating immunoglobulin concentration and disc diameter, as follows:
   a. Locate on the horizontal axis the point corresponding to the average disc diameter obtained for the 100% (undiluted) secondary standard serum.
   b. From the point on the horizontal axis, move upward vertically until reaching the level corresponding to the immunoglobulin concentration of the 100% secondary standard serum (in International Units) on the vertical axis, and make a pencil mark there.
   c. Repeat steps a and b for the 50%, 25%, and 12.5% dilutions of the secondary standard serum. There are now 4 plotted points arrayed on the paper.
   d. With a **French curve, draw** a "line of best fit" through the 4 plotted points.* Figure 2 illustrates one such line.
7. Determine the **validity** of the test, as follows:
   a. Locate on the horizontal axis of the reference line graph the point corresponding to the average disc diameter obtained for the "high" internal standard serum.
   b. From this horizontal axis point, move upward vertically until reaching the reference line. **Read** across horizontally to the vertical axis and obtain the corresponding immunoglobulin concentration. Record this result.
   c. Repeat steps a and b for the "low" internal standard serum.

*Note: In many instances the points will be linearly aligned, and a straight-edge will be satisfactory for the "line of best fit."

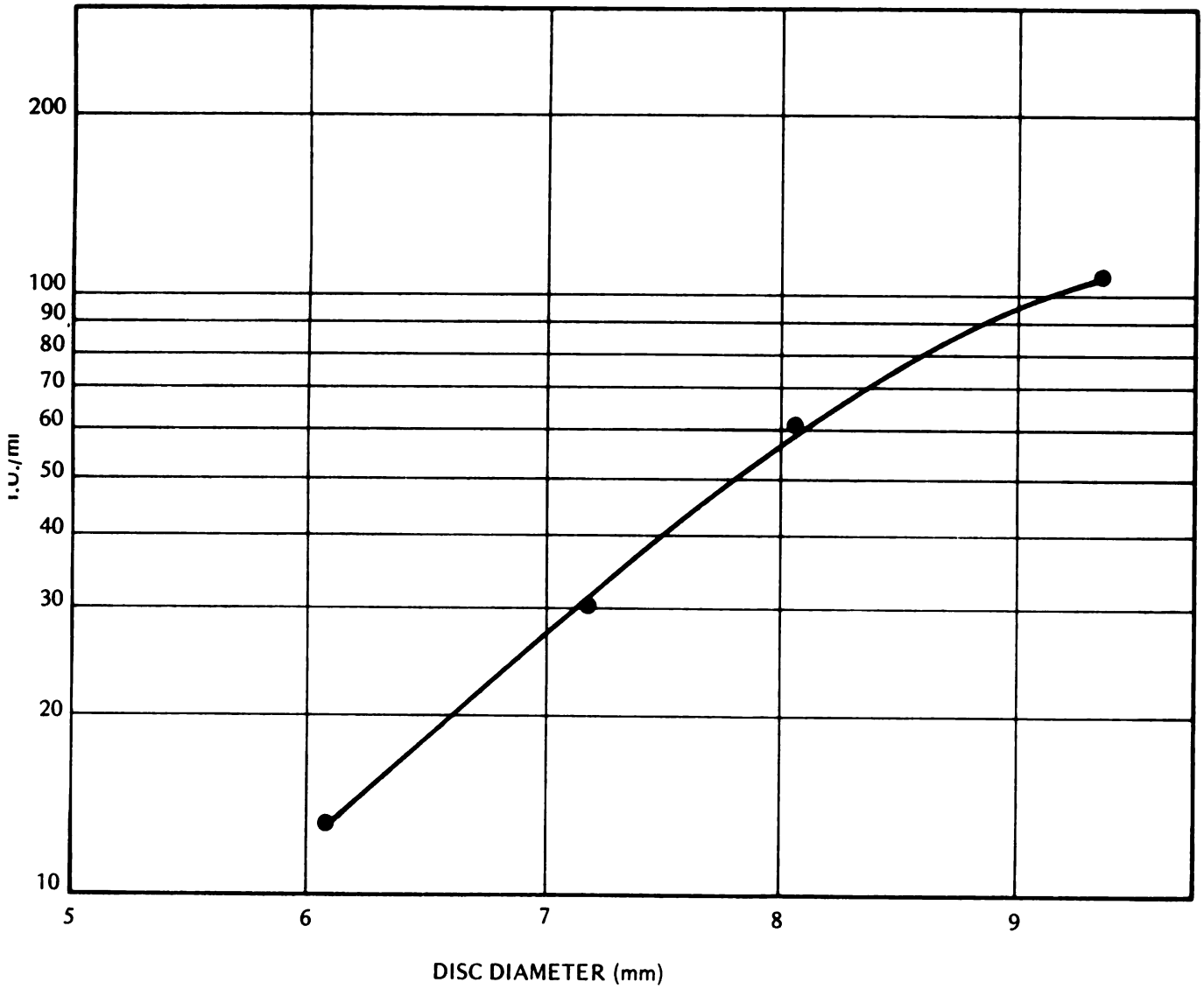
d. Plot data obtained for the internal standard sera on previously constructed quality control charts (See Quality Control Section, page 90 for construction).

If immunoglobulin concentrations for both "high" and "low" internal standard sera fall within acceptable limits (± 2 standard deviations from the mean), the test may be considered valid, and you may continue to the next step.

If one or both internal standard sera do not fall within acceptable limits on their charts, the test is invalid and must be repeated.

8. Determine the concentration of immunoglobulin in patients' sera, as follows:
   a. Locate on the horizontal axis of the reference line graph the point corresponding to the average disc diameter obtained for serum from patient #1.
   b. From this horizontal point, move upward vertically until reaching the reference line. Read across horizontally to the vertical axis and obtain the corresponding immunoglobulin concentration. Multiply the immunoglobulin concentration by 2 to compensate for dilution of the serum. Record this result.
   c. Repeat steps a and b for each patients' serum.

FIGURE 2
EXAMPLE OF REFERENCE LINE PLOTTED FROM WORKING STANDARD SERUM DILUTIONS



QUALITY CONTROL

Many variables affect the reaction between antigen and antibody in the precipitin test. Some important factors affecting the reaction are: 1) the **nature** of the antigen and antibody involved and their ratio to one another, 2) the pH, molar concentration, and ionic strength of the diluent, and 3) the temperature at which the reaction occurs.

Several other variables are introduced when immune precipitation is allowed to occur in a gel menstruum. Among these are: 1) the "pore size" of the gel (which depends on its concentration), 2) the molecular size and configuration of the antigen and antibody reactants, and 3) the sign and magnitude of the net electric change on the gel molecules. Consequently, **it is extremely important that conditions set up for the radial immunodiffusion test be carefully controlled if accurate, reproducible results are expected.**

There is no method for **absolute** determination of immunoglobulin concentration by radial immunodiffusion. Results obtained on specimens with unknown immunoglobulin concentrations must be related to results obtained with **reference standards** tested under identical condition. In the past, "reference" materials available from a number of sources contained immunoglobulins which were assayed by various methods and then expressed in terms of weight per volume (e.g., mg/ml). Unfortunately, there has been a lack of uniformity among the many reference preparations, and their use has resulted in highly discrepant values obtained on the same specimens tested in different laboratories.

The World Health Organization, recognizing the need for a reference standard with which all immunoglobulin radial immunodiffusion assays could be directly or indirectly compared, developed an **International Reference Preparation of Human Immunoglobulins IgG, IgA, and IgM (designated 67/86).** Concentrations of immunoglobulins in this material are expressed in terms of International Units (I.U.) rather than on a weight/volume basis. The WHO Expert Committee on Biological Standardization **defined an International Unit for human immunoglobulins IgG, IgA, and IgM as the activity of each of these contained in 0.8147 mg of the International Reference Preparation.** Laboratories using the International Reference Preparation as a standard have shown that comparatively good agreement among laboratories generally can be achieved in the assay of human immunoglobulins, and it is therefore recommended that all laboratories engaged in immunoglobulin quantitation relate their assays to the International Reference Preparation.

Since there is a limited supply of the 67/86 standard, it is necessary to develop "secondary" or "tertiary" standards for use as **working standards** in routine testing. Immunoglobulin preparations which have been assayed by direct comparison with the International Reference Preparation are referred to as secondary standards, and material which has been assayed by comparison with secondary standards (and thus indirectly with the International Reference Preparation) are referred to as tertiary standards. Reference material supplied by commercial companies for immunoglobulin quantitation is ordinarily secondary or tertiary standard.

The WHO Expert Committee on Biological Standardization has made a number of recommendations in regard to the use of the International Reference Preparation and expression of immunoglobulin concentration in terms of International Units. These recommendations are:

1. Concentrations of IgG, IgA and IgM in working standards, as distributed by manufacturers and others for serum estimations, should be expressed in International Units per milliliter, following direct comparison of the working standard with the International Reference Preparation or other suitably calibrated preparation.

2. Concentrations of IgG, IgA and IgM in human sera should be expressed in International Units per milliliter following comparison with the calibrated working standards.

3. To ensure continuity with previous practice, manufacturers and others should continue, for a transitional period, to indicate the estimated immunoglobulin contents of their standards by weight as well as in international units. The relationships between estimated weight and international units for a given immunoglobulin may be expected to differ between different standards; hence the relationship established for any one batch of standards should not be used as a "conversion factor" for any other standard.

4. The same principles regarding measurements and expression of serum concentrations of IgG, IgA and IgM in units should also apply to the measurement of IgD and IgE, for each of which a national research standard is now available and national units have been defined. It is hoped that at a later stage international reference preparations of these immunoglobulins will be established and international units defined.

Working standards are now available and may be purchased from commercial sources, or a working standard may be prepared in your laboratory.

Preparation of a Working Standard

1. Determine approximate quantitative values for all relevant immunoglobulin classes in the pool by routine testing (with a calibrated working standard obtained commercially or elsewhere) to make sure that the immunoglobulins are in the desired range of concentration.

2. Send 1-2 ml of the pool to an appropriate authoritative laboratory* for calibration of the pool by comparison with the International Reference Preparation.
3. If the pool is satisfactory as a working standard, prepare a minimum of three dilutions of the pool calculated to result in immunoglobulin concentrations spread over a wide range so that an accurate reference line can be established. Prepare all serum dilutions in 1% bovalbumin saline. A 100%, 50%, 25%, and 12.5% series of pool dilutions should provide an adequate range of immunoglobulin levels.
4. Prepare approximately equal volumes of each dilution; divide into convenient aliquots (e.g., 0.25 ml); and store in small vials labelled with the pool identity, dilution, and date. Store in the frozen state at −20°C or colder.

*e.g., NCI Immunoglobulin Reference Center, 6715 Electronics Drive, Springfield, Virginia 22151

INTERNAL CONTROL SERA AND QUALITY CONTROL CHARTS

The slope and position of the reference line constructed from values obtained with **four concentrations of the working standard serum pool** varies from test to test.

A convenient method of estimating the extent of that variation and monitoring test reproducibility is by means of **"internal control"** sera. These are specimens from a serum pool which are included on each slide along with the working standard dilutions, and their immunoglobulin content is determined from the reference line in the same manner as patients' sera. The internal control system should consist of one serum containing a **"high" level of immunoglobulin** close to the upper limits of the reference line and a **"low" level of immunoglobulin** close to the lower limits of the reference line.

Repeated assay of these internal control sera allows the technician to estimate the amount of variation inherent in the procedure and to construct a **"quality control chart"** consisting of the average immunoglobulin level for each internal control serum plus limits of acceptability of results from any given test. Internal control sera and their corresponding "quality control charts" are prepared as follows:

PREPARATION OF INTERNAL CONTROL SERA

1. Prepare a *pool of human sera* with normal immunoglobulin levels in a volume sufficient for repeated testing over a long period of time. This pool is used to make both "high" and "low" internal control sera.
2. Remove approximately four-fifths of the serum pool and prepare convenient aliquots (e.g., 0.25 ml) in small vials for use as "high" internal controls. Label each vial with its content, the lot number, and the date of preparation. Store at −20°C or colder.
3. Make a 1:5 dilution of the remaining one-fifth of the pool in 1% bovalbumin saline. Prepare convenient aliquots (e.g., 0.25 ml) in small vials for use as "low" internal controls. Label each vial with its content, the lot number, and the date of preparation. Store at −20°C or colder. Approximately equal volumes of each internal control serum are made in this way.

PREPARATION OF QUALITY CONTROL CHARTS

1. Under ideal, well-controlled conditions, perform approximately **30** repeated immunoglobulin determinations on each of the internal control sera. **These determinations should be done on different days, using different batches of agar, buffer, etc.**
2. Compute the arithmetic mean immunoglobulin concentration for each internal control serum as follows:

$$\text{Mean} = \frac{\text{Sum of immunoglobulin concentrations for all tests}}{\text{Number of tests performed}}$$

3. Compute the standard deviation for the mean, as follows:

$$\text{Standard deviation} = \sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n-1}}$$

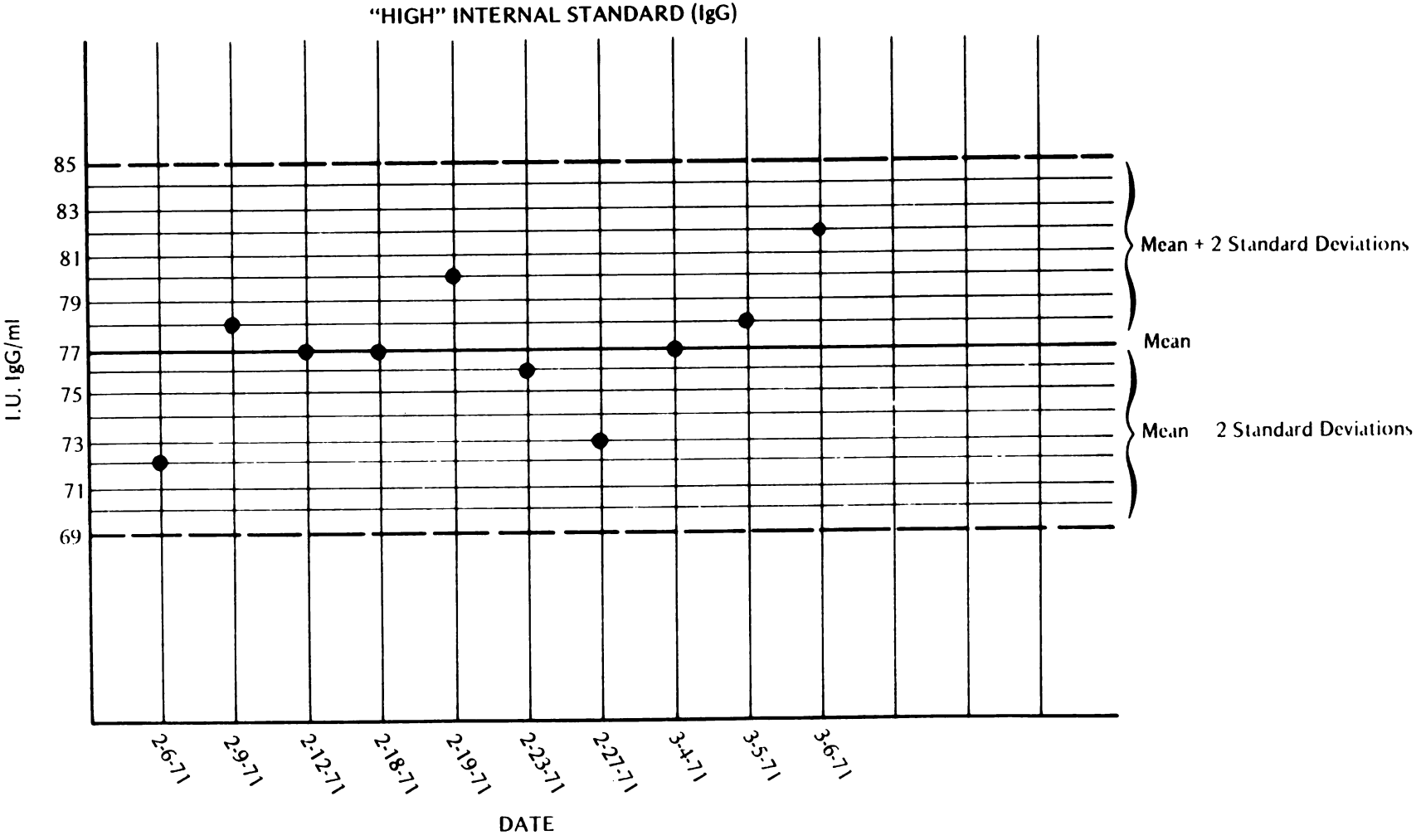
where x = individual test immunoglobulin concentration.
n = number of tests performed.

4. Construct a chart similar to that in Figure 3 for **each internal control serum and each class of immunoglobulin.** Label the vertical axis "International Units/ml Immunoglobulin (IgG, etc)" and the horizontal axis "Date." Draw a heavy solid line horizontally through the middle of the chart to represent the mean immunoglobulin concentration computed in step 2 above.

5. Draw dashed lines horizontally across the chart at the level representing the mean **plus** 2 standard deviations and at the level representing the mean **minus** 2 standard deviations. These dashed lines indicated the limits of acceptability for values obtained with the internal control serum for which the chart was constructed.

   If the value for a given internal control serum falls outside the limits of acceptability, the test should be repeated.

FIGURE 3
EXAMPLE OF QUALITY CONTROL CHART FOR INTERNAL STANDARDS



APPENDIX

PRE-COATING SLIDES WITH AGAR

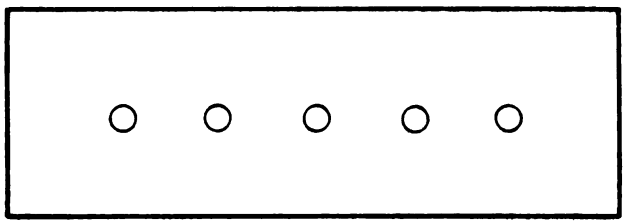
1. Clean slides with detergent; then rinse serveral times in tap water and once in distilled water. Let dry.
2. Prepare a 1% Noble agar solution in distilled water and bring to a boil.
3. Spread hot, melted agar over the entire face of the slides with a cotton swab or spread a small amount of agar across the width of the slide at one end and use the edge of another slide to spread the agar in the same manner that blood films are made.
4. Mark the underside of each slide with a wax marking pencil to identify the uncoated side and allow slides to dry overnight at 37°C.
5. Store pre-coated slides by stacking between sheets of glassine paper and wrapping the stack in aluminum foil. Store at 4°C.

WASHING, DRYING, AND STAINING AGAR SLIDES

1. Gently rinse the agar slides in distilled water, taking care to completely fill all wells with water. At the same time, soak a piece of filter paper (Whatman #1 cut slightly larger than the agar slide) in distilled water and lay it over the slide so that it completely covers the agar. Take care to avoid any air bubbles in the wells, or cracking of the agar will occur in drying.
2. Place in a 37°C incubator for overnight drying, or direct a stream of air from a warm air blower onto the slide for quicker drying.
3. When the agar is completely dry, wet the filter paper covering it with distilled water and peel off. Rinse the slide in a stream of distilled water while gently rubbing off any fuzz from the filter paper with your finger.
4. Fill a staining dish with enough phosphate buffer to completely cover the slides. Place a magnetic bar in the dish.
5. Totally immerse the slides in the phosphate buffer, place the dish on a magnetic stirrer, and regulate the speed so that good swirling occurs.
6. Wash slides for 4 - 5 hours, completely replacing the phosphate buffer 2 times during this period. If the slides contain an unusually high concentration of protein, extend the wash period to an overnight duration.
7. Rinse slides by complete immersion in distilled water for 10 minutes.
8. Fill a staining dish with 0.3% Thiazine Red R staining solution and immerse slides in stain for 10 minutes.
9. Fill a staining dish with 1% acetic acid and decolorize slides for 20 minutes, changing the 1% acetic acid 2 times during this period.
10. Fill a staining dish with 1% acetic acid - 1% glycerol solution and immerse slides in this solution for 10 minutes.
11. Allow slides to dry. They are now ready to be read.

PRE-DETERMINATION OF OPTIMAL ANTISERUM DILUTION FOR MIXING IN AGAR

1. Label three 15 x 125 mm tubes with the following antiserum dilutions: 1:10, 1:20, and 1:40.
2. Add 3.6 ml of phosphate buffer to the tube labeled 1:10, and add 2.0 ml of phosphate buffer to each of the remaining 2 tubes.
3. Add 0.4 ml of undiluted antiserum to the phosphate buffer in the tube labeled 1:10 and mix thoroughly.
4. Transfer 2.0 ml of the dilution to the tube labeled 1:20 and mix. Continue making these 2-fold dilutions through the tube labeled 1:40, discarding the last 2 ml from the last tube.
5. Place the three antiserum dilutions in the 56℃ water bath to warm up.
6. Prepare approximately 8 ml of 3% Noble agar and allow to come to 56℃ in the water bath.
7. Add 2.0 ml of the warm 3% Noble agar to each of the three antiserum dilutions and mix thoroughly. Keep the antiserum-agar mixtures in the 56℃ water bath until needed.
8. Label three 1 x 3 inch pre-coated microscope slides with the following: 1:10, 1:20, and 1:40.
9. Place the slide labeled 1:10 on a level surface.
10. Pipette 3.5 ml of the 1:10 antiserum-agar mixture onto the slide.
11. Cover the slide with one half of a petri dish to retard drying.
12. Repeat steps 9 through 11 for each of the two remaining antiserum dilutions. Let all slides stand at room temperature at least 20 minutes.
13. Using the template pictured below and a 3 mm punch, cut 5 wells, 1.2 cm apart, in each of the agar slides.



14. Remove the agar plugs from the wells by suction with a Pasteur pipette. Cover the slides and set aside.
15. Select a serum from a patient with myeloma or other pathologic concentration of the class of immunoglobulin homologous with the antiserum being evaluated.

    Make 3 serial 4-fold dilutions of the serum in phosphate buffer, starting with 1:2 and diluting through 1:32.
16. Uncover the 3 agar slides and fill wells #1 through #3 in each slide with these serum dilutions, starting with the 1:2 dilution in well #1 and ending with the 1:32 dilution in well #3.
17. Fill well #4 in each slide with **"High" Internal Standard Serum** containing the homologous immunoglobulin and fill well #5 in each slide with **"low" Internal Standard serum** containing the homologous immunoglobulin.
18. Incubate the slides in humid chambers for the times and temperatures designated in Table 1, page 83.
19. After incubation, either read slides immediately or stain with Thiazine Red R (page 95).

20. Measure diameters of all precipitin rings on all 3 agar slides and record. Also note the sharpness of the precipitin ring periphery, the presence of any internal structure (secondary rings) within the patterns, and any dissimilarity between disc appearance of the internal standard sera and the myeloma serum.

21. Determine the optimal antiserum dilution to use by the following criteria:

POTENCY: Because of the high cost of antiserum and other considerations, it is desirable to have an antiserum which can be diluted out to 1:10 (final dilution in agar) at least, and preferably much more.

Estimate the highest dilution of antiserum which, when reacting with serum containing a normal immunoglobulin level, results in a precipitin disc approximately 7 - 9 mm in diameter within the time limits of the test.

SHARPNESS:

Choose antisera (or dilutions of antisera) which give clean, sharp peripheral boundaries of the precipitin discs.

INTERNAL STRUCTURE:

Choose antisera which *do not* result in secondary rings within the patterns.

Since radial immunodiffusion is a null method of analysis in which you are attempting to match a dilution of an unknown sample with a particular dilution of a "known" sample to give a precipitin disc of identical size, the two samples should appear identical in all other respects as well. Some antisera will cause the two to appear different and should be rejected.

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G. Antistreptolysin O Test

Antistreptolysin O Test

Hemolytic streptococci occupy a pre-eminent position of importance in human disease. These organisms are involved in acute infections such as upper respiratory disease, scarlet fever, puerperal fever, erysipelas, as well as in delayed sequelae such as rheumatic fever, glomerulonephritis, erythema nodosum, and rheumatoid arthritis.

Serologic studies are of great importance in the immunology of rheumatic disease. The Antistreptolysin O test is often used to aid in the diagnosis of rheumatic fever, glomerulonephritis, and other disease states.

1. **Principles**

   The serologic test for detecting antistriptolysin O (ASO) is based on the following principles:

   a. Streptolysin O reacts enzymatically with erythrocytes to bring about their lytic dissolution.

   b. ASO specifically neutralizes streptolysin O, resulting in an inhibition of its hemolytic activity.

   c. The amount of ASO present in a patient's serum can be determined by adding constant volumes of streptolysin O to several dilutions of serum. The ASO titer is an expression of the serum dilution containing just enough ASO to completely prevent hemolysis.

2. **Materials**

   a. **Reagents**

      1) Gelatin-barbital buffer
      2) Cold distilled water
      3) Ice
      4) Streptolysin O
      5) 2.5 percent suspension of sheep or rabbit red blood cells
      6) Standard antiserum
      7) Test sera

   b. **Equipment**

      1) Microtitration equipment

         Calibrated pipette droppers. One 0.05 ml and two 0.025 ml per person
         Calibrated microdiluters. Six 0.05 ml per person
         U plates (disposable or Lucite). One plate per six specimens, one cover plate
         Microdiluter testers (Go-No-Go) 0.05 ml
         Cotton swabs
         Centrifuge carriers
         Test reading mirror
         Stand for microdiluters and pipettes
         Vibratory mixer

      2) Incubator (37°C)
      3) Centrifuge. International Size 2 with No. 976 head
      4) Serological pipettes (0.2 ml, 1.0 ml, and 2.0 ml)
      5) Test tubes (15 x 85 mm or 13 x 100 mm)
      6) Test tube supports

7) Interval timer
8) Burner
9) Beakers
10) Cleansing tissues
11) Fine tip felt marking pen
12) Pink fluorescent lamps, Fadex

3. **Methods**

a. Preparation of reagents and materials

1) Preparation of gelatin-barbital buffer (GB)

a) Stock barbital buffer solution

Barbital (diethylbarbituric acid)	3.3 g
Sodium barbital (Sodium diethylbarbiturate)	1.4 g
Sodium chloride	42.5 g
Distilled water, qs to	1000 ml

Add the barbital and sodium barbital to approximately 500 ml of distilled water. Heat to just below boiling with constant stirring until the barbital dissolves. Add sodium chloride; mix thoroughly until sodium chloride dissolves. Remove from heat and add approximately 400 ml of distilled water. Cool the solution to room temperature. Transfer to a 1000 ml volumetric flask and bring to volume with distilled water. Determine the pH. It should be 7.2 ± 0.05. Store at 4°C. Discard at first sign of contamination.

b) Stock gelatin solution

Gelatin 1.25 g
Distilled water qs to 1000 ml

Add the gelatin to approximately 200 ml of distilled water. Heat to boiling with constant stirring to prevent scorching the gelatin. Remove from heat and add approximately 300 ml distilled water. Cool to room temperature. Transfer to 1000 ml volumetric flask and bring to volume with distilled water. Store at 4°C. Prepare **only** enough gelatin water to last 7-10 days, as it is easily contaminated. **Discard** at first sign of bacterial or mold growth.

c) Working solution of GB

Prepare a working solution as needed by adding 1 part of stock barbital solution to 4 parts of gelatin solution, e.g. 20 ml of buffer solution to 80 ml of gelatin solution. Store in refrigerator. Discard at first sign of contamination

2) Preparation of 2.5 percent red blood cell suspension

a) Pipette 3-4 ml of citrated rabbit or sheep blood through two layers of clean gauze into a 15 ml graduated centrifuge tube. Add two or three volumes of GB to each volume of blood. Centrifuge at 600 x g **for 5 minutes.**

b) Remove the supernatant and the layer of white blood cells. Fill the centrifuge tube with GB. Resuspend the cells by mixing gently with a pipette. Centrifuge at 600 x g for 5 minutes. **Repeat the washing process two times.** If the supernatant is not colorless after two washings, the cells are too fragile and should not be used.

c) After the second washing, add GB to the packed cells up to the 10 ml graduated mark. Resuspend the cells with a pipette. Centrifuge at 600 x g for **10 minutes.** Record the column of packed cells and remove supernate. Prepare a 2.5 percent suspension by adding 3.9 ml of buffered diluent to each 0.1 ml of packed cells.

d) Store the cell suspension at 4°C. Discard at the first sign of hemolysis or contamination.

3) Preparation of initial serum dilutions

a) The **initial** serum dilutions of 1:10, 1:60, and 1:85 are prepared in **test tubes.** Subsequent dilutions are carried out in the U plates. Both the serum and GB should be at room temperature when preparing the dilutions. Include a standard serum of known titer in each day's run to serve as a positive control.

b) Prepare dilutions as shown below. Mix thoroughly.

Tube Number	Initial Dilution	Amount GB	Amount Undiluted Serum	Amount 1:10 Serum
1	1:10	0.9 ml	0.1 ml	-----
2	1:60	1.0 ml	-----	0.2 ml
3	1:85	1.5 ml	-----	0.2 ml

b. Performance of Test

1) Label a microtiter "U" plate as indicated in the plate pattern so that each specimen is assigned two rows (1:60 row and a 1:85 row), with six wells per row.

Label six wells on the bottom row of each plate as specimen hemolysin controls. Also label a well for the streptolysin control and a well for the RBC control on one of the plates in each run **(not for each plate).**

2) Add GB, at room temperature, as follows:

0.05 ml (0.05 ml dropper) to wells 2 through 6 of each row assigned to specimens, and to the streptolysin 0 control well.

0.025 ml (0.025 ml dropper) to each serum hemolysin control well.

0.075 ml (0.025 ml dropper) to the RBC control well.

3) Pipette (0.2 ml pipette) 0.1 ml of each 1:60 serum dilution into the labeled test well.

4) Pipette 0.1 ml of each 1:85 serum dilution into the labeled test well and 0.05 ml into the serum hemolysin control wells.

5) Test an 0.05 ml microdiluter for accuracy.

6) Transfer 0.05 ml from the first well to the second well.

7) Prepare serial twofold dilutions through the sixth well. The serum dilutions for each serum are: **First row** - 1:60, 1:120, 1:240, 1:480, 1:960, 1:1920; **Second row** - 1:85, 1:170, 1:340, 1:680, 1:1360, and 1:2720.

8) Check the microdiluters for accuracy and place in distilled water for rinsing.

9) Repeat any dilution series which gives an inaccurate diluter check.

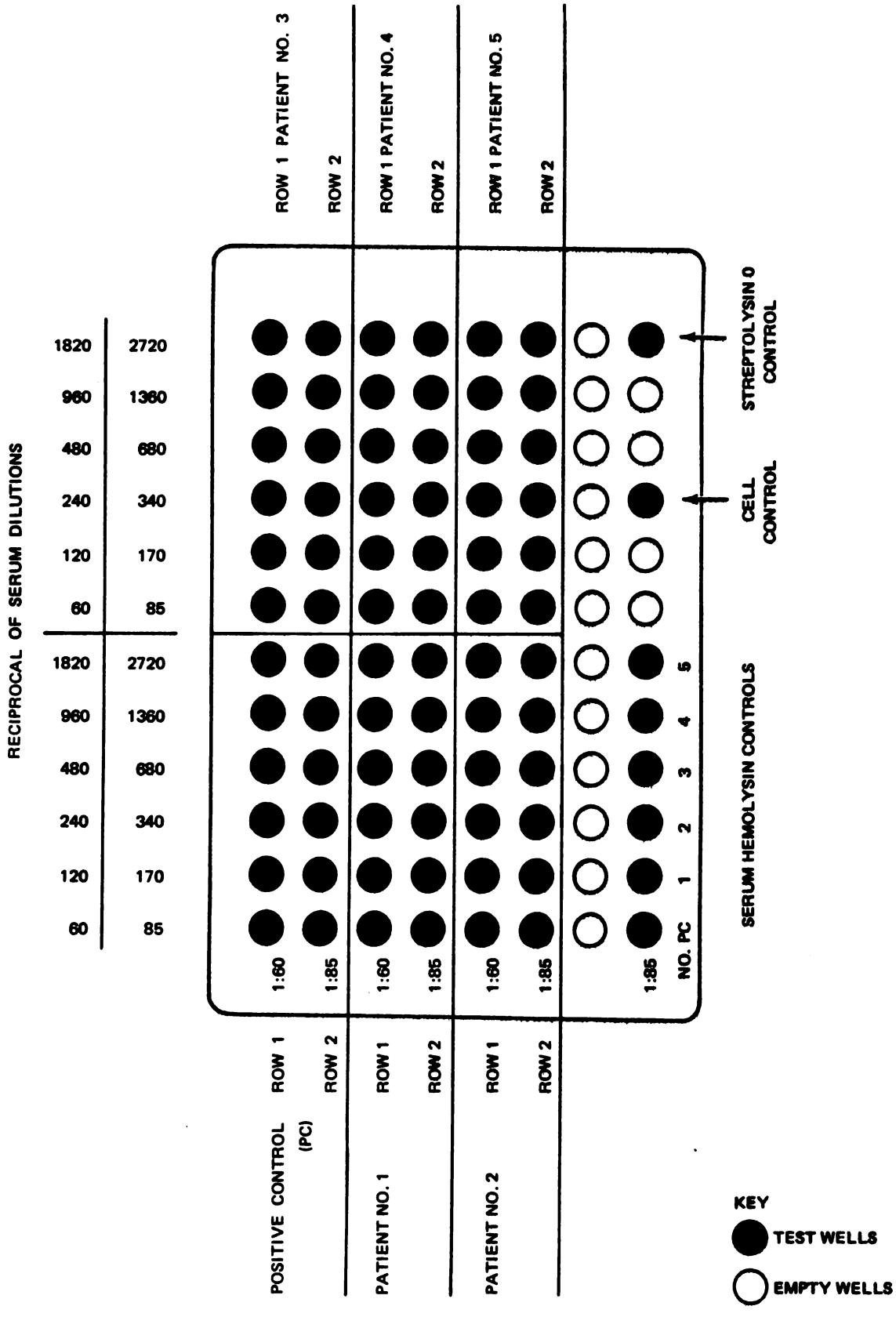
10) Reconstitute the streptolysin 0 reagent according to package directions with **COLD** distilled water. Mix **gently** to avoid aeration. Keep the reconstituted reagent in ice water.

11) Add 0.025 ml of cold streptolysin 0 reagent to all wells **except** the RBC and serum hemolysin controls.

12) Turn on vibrator.

13) Place plate on vibrator for 20 seconds. Remove plate **before** stopping vibrator. **Caution: extended** agitation may cause oxidation and inactivation of the streptolysin 0.

ASO TITRATION PLATE PATTERN



14) Cover plate with an empty plate. If there is more than one test plate, 3 plates may be stacked and the cover placed on top.

15) Place covered plate in a 37℃ incubator for 15 minutes.

16) Remove plate from incubator and add 0.025 ml (0.025 ml dropper) of cold 2.5% red blood cells to **all** wells. Do not add RBC to more than 6 plates at a time before mixing on the vibrator because the RBC will settle out and be more difficult to resuspend.

17) Mix on vibrator for 15-20 seconds or until all cells are in suspension.

18) Restack and cover plates.

19) Incubate at 37℃ for 15 minutes.

20) Remove plates from incubator.

21) Mix on vibrator for 15-20 seconds or until all cells are in suspension.

22) Restack and/or cover plates.

23) Reincubate at 37℃ for 30 minutes.

24) Centrifuge plates for 2 minutes at 250 g to pack the RBC (1200 rpm in size 2 International Centrifuge with No. 976 head).

25) Read for presence or absence of hemolysis, using a reading mirror and fluorescent lamp.

4. Reading of Test

a. Examine streptolysin control for **presence** of **complete hemolysis**

b. Examine **cell control** for *absence* of *hemolysis.*

c. Examine **serum control** for *absence* of *hemolysis.*

d. Validity. The test must be repeated on all sera in the run if:

1) The titer of the reference serum is not as stated by the manufacturer.

2) The streptolysin 0 control does not show complete hemolysis of the RBC.

3) The RBC control shows any *hemolysis.*

e. If there is hemolysis in the serum hemolysin control, the test on that specimen is not valid unless there is at least one well above the 1:85 dilution in the test (for that particular serum) which has no hemolysis. Hemolysis in a serum hemolysin control well indicates the presence of a natural hemolysin for the RBC in the patient's serum. This hemolysin is usually diluted out at dilutions above 1:85, and does not interfere with the determination of the endpoint.

5. Reporting Results

The titer may be expressed in Todd Units (T.U.) if the potency of the streptolysin 0 used in the test had been adjusted against the Todd standard, *or* in International Units (I.U.) if the potency of the streptolysin 0 used in the test had been adjusted against the WHO International Standard. For practical purposes, these two units are equivalent.

6. Precautions and Helpful Suggestions

a. Streptolysin

1) Avoid shaking or aeration of the streptolysin solution. Mix **gently** when reconstituting. Oxidation reduces the hemolytic activity.

2) Leftover streptolysin solution must be discarded. It is not reusable.

b. **Red blood cell suspension**

1) Do *not* use the same pipette dropper for red blood cells that was used for streptolysin solution. A trace of streptolysin in the dropper can cause some lysis of the cells.

2) Do **not** add cells to more than 6 plates at a time before mixing the plates; the cells settle out and are difficult to resuspend.

c. **Incubation**

1) To ensure a proper incubation temperature for all plates, do not put more than three plates (plus cover) in a stack.

2) Keep a pan of water in the incubator to provide a moist atmosphere to prevent evaporation from the plates.

d. **Vibrator**

1) Syntron paper jogger (or equivalent) or Thomas Shaking apparatus is satisfactory.

7. **Interpretation of Results**

a. The most convincing serological evidence suggestive of a recent group A streptococcal infection is a rise in titer of two dilution steps or more between acute and convalescent phase sera. A rise in titer occurs about one week after infection and peaks two to four weeks later. In the absence of complications or reinfection, the ASO titer will usually fall to preinfection levels within 6 to 12 months.

b. If only a single specimen is available, or in the absence of a two dilution rise (or fall) in titer, then the upper "limit of normal" titer is a useful guide for determining the significance of the ASO titer.

1) The upper "limit of normal" value represents the highest ASO titer obtained in 85% of a "normal" population with no apparent recent streptococcal infection. Thus, an ASO titer above the upper limit has an 85% chance of being due to a recent streptococcal infection.

2) It should be recognized that an ASO titer of a single specimen below the upper "limit of normal" cannot definitely rule out a streptococcal infection since a significant increase in titer (twofold or more dilutions) may occur even though the titers do not exceed the "normal" range.

c. The upper "limit of normal" for ASO titers varies with age and may also vary with geographic location.

1) The upper limits of normal ASO titers in young adults is in the range of 200. Approximately 80% of this population will have titers below the 200 level. Younger people from 6-14 years of age may have somewhat higher titers; the upper limit of normal for this age group is approximately 250. Normally, as age increases above 17 years, the ASO titer falls eventually to a level below 100.

d. Over 80% of patients with acute rheumatic fever and over 95% of patients with acute glomerulonephritis have elevated ASO titers. These titers have been reported to range between 159 and 1585 or higher.

e. False "positive" ASO titers can be caused by increased levels of beta lipoproteins in serum due to liver disease, by contamination of the sera by *B. cereus* or with several species of *Pseudomonas*, and by oxidation of the streptolysin 0.

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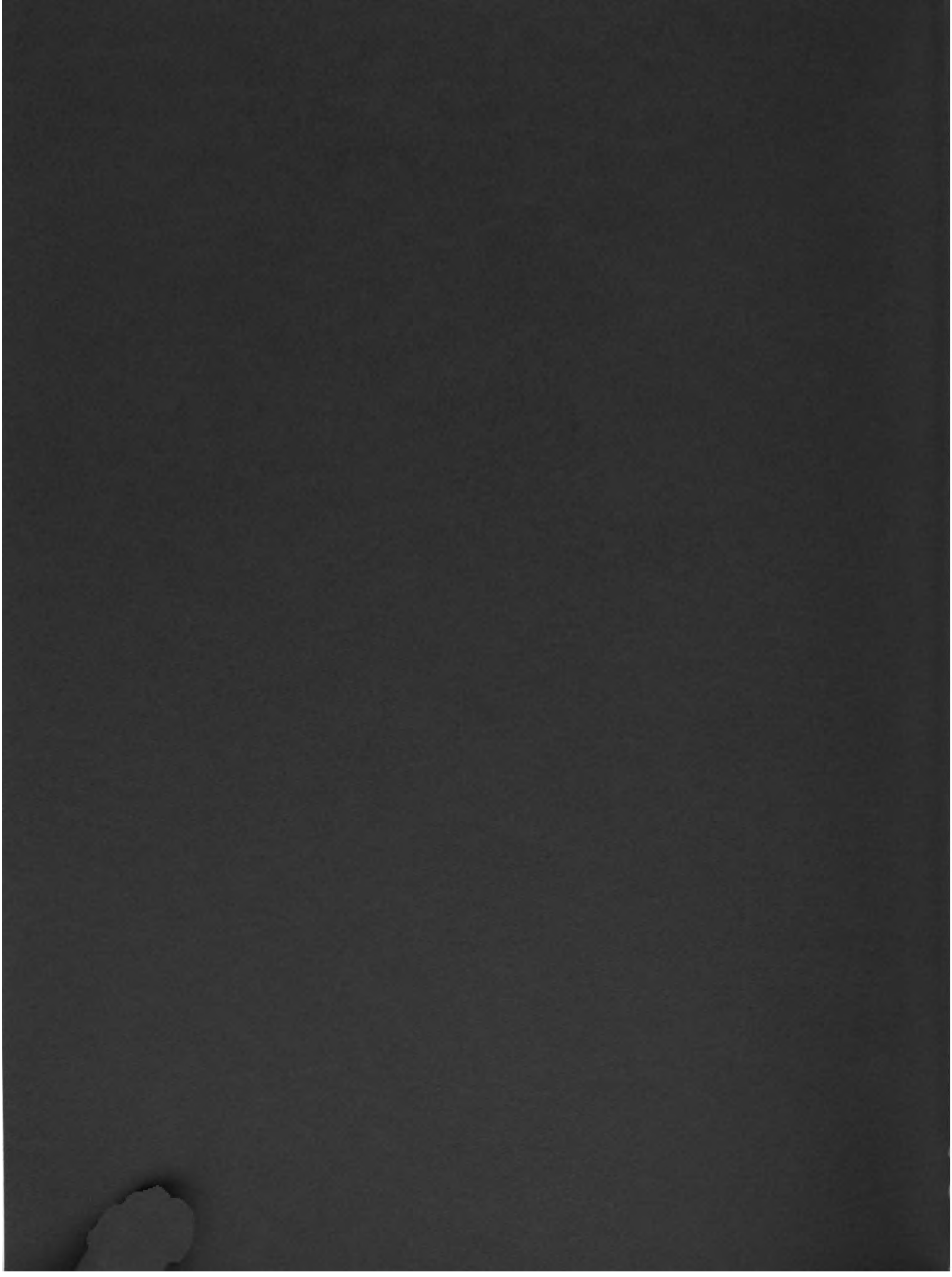


AUTOMATION OF SEROLOGIC PROCEDURES

by Kenneth W. Walls, Ph.D.

Automation in serology is still very much in its infancy. Some prefer to call it "mechanization," but it has progressed past that. Microtitration procedures were, in one respect, the first mechanization steps in serology; however, they are still very manual. In recent years a variety of instruments have been developed which mechanize and automate these microtitration methods; each has its use.

Several devices which drop diluent in the 96 wells of a microtitration plate are available. These are particularly useful where a number of different reagents or procedures are being performed simultaneously. These instruments are relatively inexpensive, accurate, and rapid. They are compact and easily moved where needed; they can be sterilized, if necessary, and can be used conveniently in biological safety cabinets or similar protective areas. These advantages must be weighed against the following disadvantages: (1) that, within reason, only one reagent can be dropped with each filling; (2) each plate must be manually placed into the instrument and removed from it; (3) mechanical shaking to mix reagents is necessary.

Similarly, a number of instruments have been devised to operate the diluting loops. These vary from hand-held motor driven loops to instruments that completely perform the dilution process. We are particularly concerned with the latter type. Several instruments of this type are available and are of similar usefulness and value. Some move the plates forward for each progressive dilution, while others move the loops over the plate. This is of minor importance since both perform the dilutions effectively. These instruments have both advantages and disadvantages. Speed of operation and lack of operator fatigue are of utmost importance. Further, all of these devices are relatively small and permit easy moving, as necessary; they may be used in protective areas and can be sterilized, if needed. The major disadvantage again is that manual manipulations are necessary. In each case, the reagent to be diluted must be added manually to the plate, and the plate then manually placed on the instrument and removed when completed.

Finally, instruments are now available which combine all of these functions simultaneously. One such device includes a plate loader which permits stacking 20 plates into the machine and requires no further manipulation until the next 20 plates are added. The reagents are added by manifold and only a single reagent can be added with each manifold. Another instrument, while not having the plate stacking device, has the capability of adding 8 different reagents from each of the 3 manifolds. The advantages and disadvantages are apparent. If only one procedure is being used to test against one antigen, the plate stacking capability is extremely useful. On the other hand, if one is testing with a battery of antigens, the ability to add 8 different reagents from a manifold is important. In both cases, certain manual manipulations are necessary: (1) the reagent to be diluted must be added manually to the plates; (2) the plates must be placed in the machine (or tray stacker) and removed manually. Although they can be sterilized, these instruments are not as small as the individual components and thus do not lend themselves to operation in a safety-cabinet.

All of the instruments discussed thus far require manual reading and recording of results. Most conventional procedures can be performed including: agglutination, hemagglutination, passive hemagglutination, and complement fixation. Reagent usage is the same as for manual microtitration tests except that in a few circumstances, particularly where labile reagents are used, a 10 percent excess must be allowed for manifold filling and cannot be reclaimed.

Another approach to automation does not utilize the microtitration principle but instead uses discrete sampling and continuous addition of reagents. This procedure has found its best use in the VDRL test for syphilis; it is also being used by a number of laboratories for complement fixation. This procedure most nearly accomplishes automation. The test sera are placed in a sample tray and all further additions are made automatically. The results are read photometrically and printed either digitally or on a line graph. A permanent record is thus available and no technical manipulations are required except between runs when the machine must be cleaned. The obvious advantage of this instrument is its total automation and the freedom it allows the operator. Several disadvantages are also apparent: (1) only a single antigen can be added per run, as even the most sophisticated model makes only two dilutions per test; (2) reagents run continuously and even though quantities are smaller than those of microtitration tests, there is still a waste of about 50 percent of each reagent; (3) greater technical expertise is required.

There are other automated systems in immunoserology in addition to those that have been mentioned. Instruments are now available for blood typing and for the determination of some immunoglobulins. The techniques and approaches are numerous but appear to be successful and useful.

Many refinements are still necessary before complete automation is available to the serologist; however, significant advances have been made. Though we have only begun, we must admit that automation in serology is here.

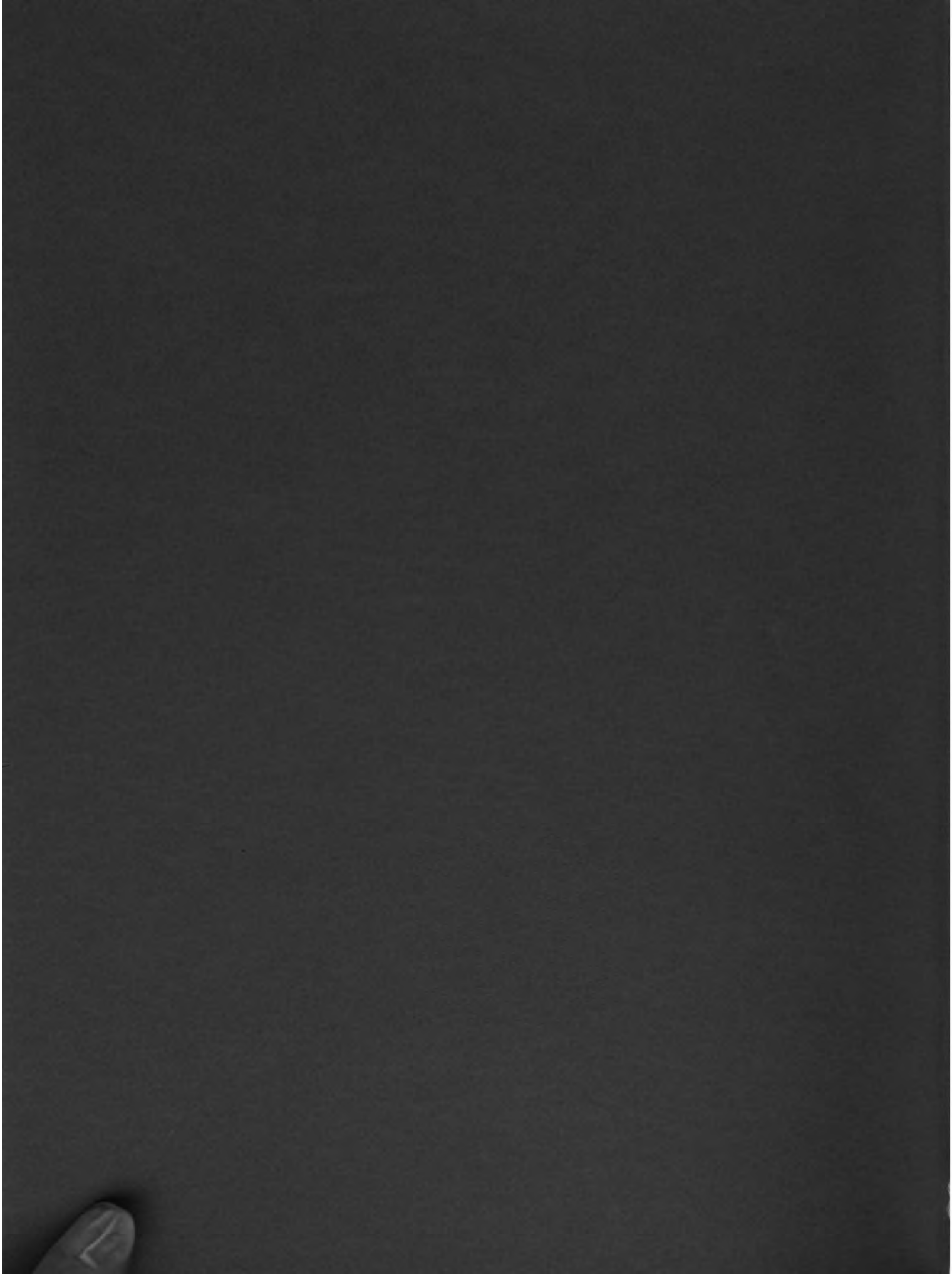
A Partial List of Major Manufacturers

Cooke Engineering Company
900 Slaters Lane
Alexandria, Virginia 22314

Technicon Instruments Corporation
Tarrytown, New York 10591

Canalco
5635 Fisher Lane
Rockville, Maryland 20852

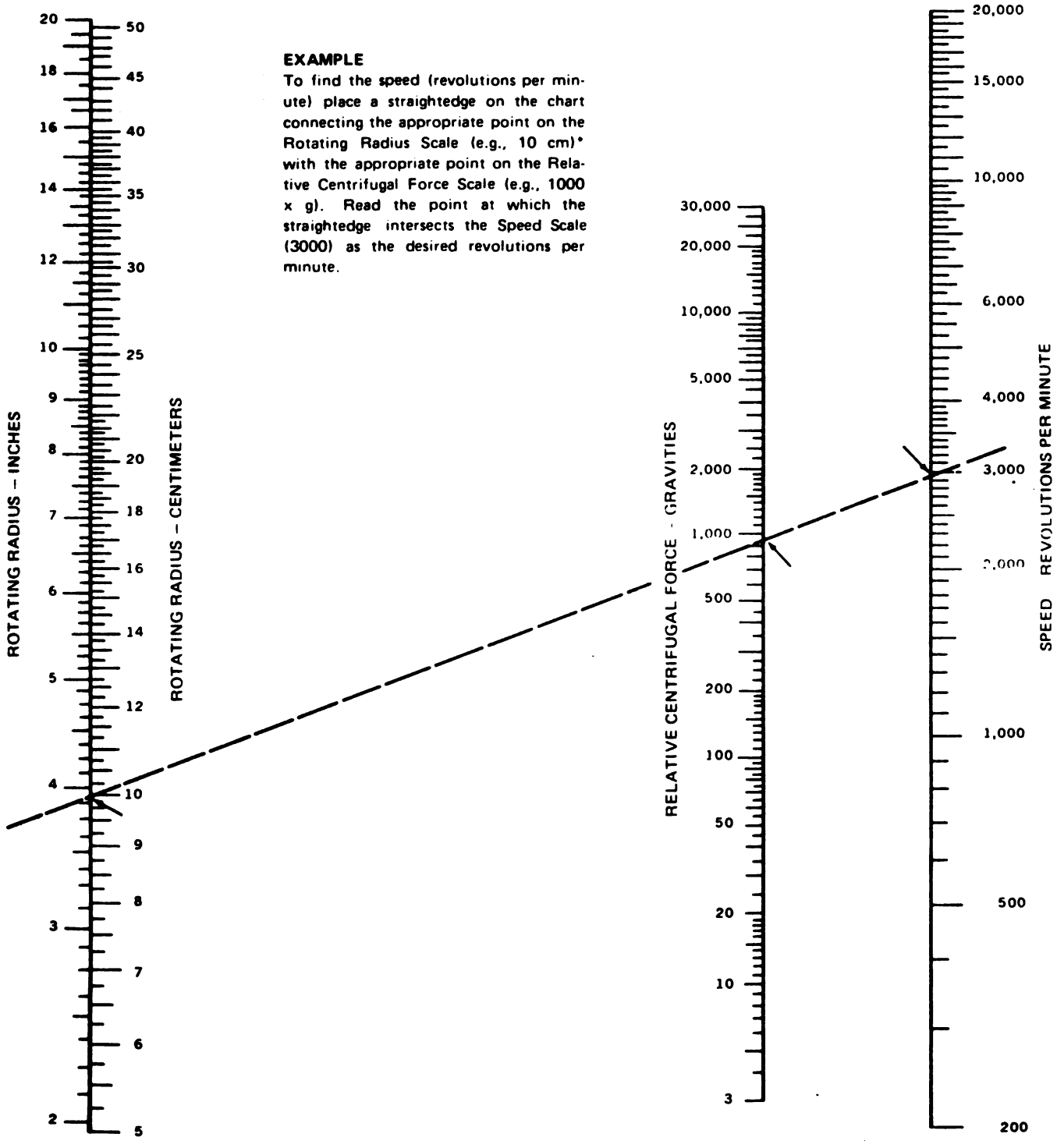
Linbro Chemical Co., Inc.
681 Dixwell Avenue
New Haven, Connecticut 06511



Information and Precautions for Performance of Serologic Tests

1. **General**
   a. Follow all protocols exactly.
   b. Titrate materials according to recommendations.
   c. Keep reagents and tests at recommended temperatures.
   d. Always read controls before reading tests; do not read tests unless all controls are correct.
   e. Check temperatures on heating and cooling equipment before use.
2. **Sera**
   a. Inactivate according to protocol.
   b. Do not use contaminated sera in tests.
   c. Always check to see whether pretreatment of sera is required.
   d. Store sera at −20°C or below.
   e. Do not freeze and thaw serum specimens more than once.
3. **Preparation of Erythrocyte Suspension**
   a. Mix the cell suspension well but gently prior to use. Check to see that no cells adhere to the bottom of the flask. As cells are added to the test, keep them evenly suspended by frequent gentle agitation.
   b. Standardization by centrifugation
      1) Be sure all glassware is carefully checked for accurate calibration.
      2) Use recommended speeds and times for washing and packing.
      3) Use proper diluent and measure carefully.
      4) Make standard suspensions by using standardized packing tubes.
      5) Read the level of packed cells carefully.
   c. Standardization by spectrophotometry
      1) Be sure instrument is used correctly.
      2) Calibrate cuvettes so a matched set is available.
      3) Store reagents properly and as recommended by manufacturers.
4. **Preparation of Buffers and Reagents**
   a. Weigh ingredients accurately on a standardized and sensitive balance.
   b. Use volumetric flasks and thoroughly dissolve reagents before bringing the volume to the proper level.
   c. Use a pH meter which has been standardized.
   d. Follow storage instructions strictly. Store all reagents in sterile, tightly stoppered bottles.
   e. Sterilize by Millipore filtration unless autoclaving is specified.
   f. Either distilled or demineralized water may be used in making up reagents, except where otherwise noted.

NOMOGRAPH FOR CALCULATING CENTRIFUGE SPEED



*To determine the rotating radius of the centrifuge head, measure – with the tube in the extended position – from the center of the drive shaft to the middle of the liquid column you are centrifuging.

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